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(57) Abstract

The present invention is directed to novel inhibitors of an HIV or other lentiviral or retroviral protease which are capable of inhibiting HIV or other lentiviral or retroviral replication. As such, these inhibitors are capable of reducing, eliminating or preventing viral infection. The inhibitors are peptides and polypeptides having an amino acid sequence which corresponds to the amino acid sequence of a lentiviral Vif protein (Vif–derived protease inhibitor). The invention is also directed to compositions containing the inhibitors of the invention and to methods for using such compositions in the prevention or treatment of HIV or other lentiviral and retroviral infections. The invention is also directed to methods for identifying such inhibitors in cellular model systems, HIV–infected cells, *in vitro* high flux assay systems, and animal model systems.

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Description

Vif-Derived HIV Protease Inhibitors

Introduction

The present invention is directed to novel inhibitors of an HIV or other lentiviral or retroviral protease which are capable of reducing, eliminating or preventing an HIV or other lentiviral or retroviral infection. The inhibitors are peptides and polypeptides having an amino acid sequence which corresponds to the amino acid sequence of a lentiviral Vif protein (Vif-derived protease inhibitor). The invention is also directed to compositions containing the inhibitors of the invention and to methods for using such compositions in the prevention or treatment of HIV or other lentiviral and retroviral infections. The invention is also directed to methods for identifying such inhibitors in cellular model systems, HIV-infected cells, in vitro high flux assay systems, and animal model systems.

Background of the Invention

Human immunodeficiency virus type 1 (HIV-1) contains several genes that encode auxiliary proteins, which are not found in oncogenic retroviruses, but which influence HIV-1 replication (Cullen, Annu. Rev. Microbiol. 45:219-250, 1991). One such protein is Vif (virion infectivity factor), a 23 kDa polypeptide which is conserved in lentiviruses (Oberste et al., Virus Genes 6:95-102, 1992; Lee et al., Science 231:1546-1549, 1986) and among HIV-1 infected persons (Kan et al., Science 231:1553-1555, 1986; Sova et al., J. Virol. 69:2557-2564, 1995). Antibodies to Vif are found in HIV-1 infected individuals, and increasing levels of Vif can be a marker of the progression of the viral infection (Schwander et al., J. Med. Virol. 36:142-146, 1992).

Vif is required for productive HIV-1 infection of primary blood lymphocytes (PBL) and macrophages in vitro (Chowdhury et al., J. Virol.

70:5336-5345, 1996; Courcoul et al., J. Virol. 69:2068-2074, 1995; Gabuzda et al., J. Virol. 66:6489-6495. 1992; Gabuzda et al., J. Acq. Imm. Def. Synd. 7: 908-915, 1994; Michaels et al., AIDS Res. and Hum. Retrovir. 9:1025-1030, 1993; Simm et al., J. Virol. 69: 4582-4586, 1995) and for in vivo pathogenesis in the SCID-hu mouse model of HIV-1 infection (Aldrovandi et al., J. Virol.70:1505-1511, 1996).

In the absence of Vif, HIV-1 infected nonpermissive cells produce noninfectious virions (Borman et al., J. Virol. 69:2058-2067, 1995; Chowdhury et al., J. Virol. 70:5336-5345, 1996; Courcoul et al., J. Virol. 69:2068-2074, 1995; Fisher et al., Science 237: 888-892. 1987; Simm et al., J. Virol. 69: 4582-4586, 1995; Von Schwedler et al., J. Virol. 67:4945-4955. 1993). Such a virus can enter cells, but is unable to efficiently synthesize viral DNA (Chowdhury et al., J. Virol. 70:5336-5345, 1996; Courcoul et al., J. Virol. 69:2068-2074, 1995; Von Schwedler et al., J. Virol. 67:4945-4955, 1993; Sova et al., J. Virol. 67:6322-6326, 1993), displaying a 2,500-fold decrease in the number of reverse transcripts compared to wild-type virus when assayed in primary macrophages (Chowdhury et al., J. Virol. 70:5336-5345, 1996).

Virions produced in the absence of Vif have aberrations in the composition of core proteins (Borman et al., J. Virol. 69:2058-2067, 1995; Simm et al., J. Virol. 69:4582-4586, 1995) and in core morphology as determined by electron microscopy (Borman et al., J. Virol. 69:2058-2067, 1995; Hoglund et al., Virology 201: 349-355, 1994). Virions assembled in the absence of Vif in primary T lymphocytes carry unprocessed Gag polyproteins, which appears to correlate with their highly attenuated infectivity (Simm et al., J. Virol. 69:4582-4586, 1995). These findings suggest that one mechanism of action of Vif is to ensure proper processing or assembly of HIV-1 core components in the production of mature infectious virions. Particles produced in the absence of functional Vif (ΔVif HIV-1) can initiate efficient infection only in certain T lymphoid cell lines which can complement Vif function, such as SupT 1, but cannot in peripheral blood

lymphocytes, which do not complement Vif function (Gabuzda et al., J. Virol. 66:6489-6495, 1992; Simm et al., J. Virol. 69:4582-4586, 1995).

HIV-1 core components are synthesized as two precursor polyproteins, $Pr55^{Gag}$ and $Pr160^{Gag\text{-}Pol}$, which consist of both overlapping and distinct polypeptides (Hunter et al., Sem. in Virol. 5:71-83, 1994; Willis et al., AIDS 5:639-654, 1991). The Gag gene encodes the core proteins p17 matrix (MA), p24 capsid (CA), nucleocapsid (NC), $p6^{Gag}$, and two spacer peptides, p2 and p1. In addition to structural proteins, $Pr160^{Gag\text{-}Pol}$ contains the viral protease (PR), reverse transcriptase, and integrase enzymes derived from the Pol gene. Both $Pr55^{Gag}$ and $Pr160^{Gag\text{-}Pol}$ are processed to their mature forms present in infectious virions exclusively by viral protease (Dougherty et al., Microbiol. Rev 57:781-822, 1993; Hunter, Seminar in Virol. 5:71-83, 1994; Willis et al., AIDS 5:639-654, 1991).

The HIV protease, one of four enzymes encoded by the virus, is an aspartic protease having a molecular weight of about 11 kDa, and containing 99 amino acids (Katz et al., Annu. Rev. Biochem. 63:133-173, 1994). The three-dimensional structure of the enzyme has been determined (Navia et al., Nature 337:615-620, 1989; Miller et al., Nature 337:576-579, 1990). Enzymatic activity requires dimerization of protease monomers to provide a functional enzymatic active site (Dougherty et al., Microbiol. Rev 57:781-822, 1993). The HIV-1 protease cleaves the Gag and Gag-Pol precursor polyproteins at at least ten distinct cleavage sites, each having a distinct amino acid sequence (Debouck et al., Drug Development Research 21:1-17, 1990). Domains adjacent to the cleavage sites in both Gag and Pol regulate protease-mediated proteolysis in cis (Goodbar-Larsson et al., Virology 206:387-394. 1995; Pettit et al., J. Virol. 68: 8017-8027, 1994; Quillent et al., Virology. 219:29-36, 1996; Zybarth et al., J. Virol. 69: 3878-3884, 1995), but no other viral or eucaryotic proteins have been shown to affect protease activity. However, the apparent similarity of the defective viral cores containing unprocessed Gag proteins produced in the absence of protease and in the absence

of Vif suggests that Vif is required for proper processing of virion protein components by protease.

Many antivirals, which have been developed against enzymatic targets in the HIV virion, have been directed to reverse transcriptase (RT), such as azidothymidine, dideoxycytosine and dideoxyinosine (Richman et al., Science 272:1886-1887, 1996). However, prolonged treatment with RT inhibitors has been associated with the development of drug-resistant mutants. Accordingly, there has been a trend away from monotherapy (use of one antiviral compound) and toward the development of combination therapies that reduce the potential for the generation of drug-resistant viral mutants.

The HIV protease has been the target for several antiviral compounds that have shown therapeutic promise, such as saquinavir, indinavir and ritonavir (Richman et al., Science 272:1886-1887, 1996). Protease inhibitors have been used in combination therapies with RT inhibitors. Most protease inhibitors developed to date have been based on classical substrate or transition-state analogue approaches (Roberts et al., Science 248:358-361, 1990; McQuade et al., Science 247:454-456, 1990). and are peptidomimetic compounds.

The recent use of protease inhibitors in clinical treatment has documented the emergence of drug-resistant HIV-1 mutants which are no longer susceptible to one or more structurally diverse inhibitors with common targets, such as indinavir (Condra et al., Nature 374:569-571, 1995). Therefore, there is a critical need for the development of novel protease inhibitors which can be used in the prevention or treatment of HIV or other lentiviral and retroviral infections.

Summary of the Invention

The present invention is directed to novel inhibitors of an HIV or other lentiviral or retroviral protease which are capable of inhibiting HIV or other lentiviral or retroviral replication. As such, the inhibitors are also capable of reducing, eliminating or preventing viral infection. The inhibitors are peptides and

polypeptides having an amino acid sequence which corresponds to the amino acid sequence of a lentiviral Vif protein (Vif-derived protease inhibitor). The invention is also directed to compositions containing the inhibitors of the invention and to methods for using such compositions to prevent, treat, or inhibit HIV or other lentiviral and retroviral infection. The invention is also directed to methods for identifying such inhibitors in cellular model systems, HIV-infected cells, in vitro high flux assay systems, and animal model systems.

Brief Description of the Drawings

The present invention may be better understood by reference to the drawings of which

Figure 1 shows the structure of constructs used to express autoproteolytic Gag-PR fusion proteins in bacteria.

Figure 2 shows the structure of constructs used to express Vif and control proteins as fusions with GST.

Figure 3. Panel A shows a schematic diagram of the Gag-PR cleavage products based on known PR cleavage sites (arrows) and their detection by the HIV-1 antisera. Panel B shows a Western blot of cells expressing Gag-PR with GST-Vif (V) or GST-N'terminal half of Vif (N'V), GST (G) or GST-C'terminal half of Vif (C'V). T cells+HIV: extracts of CR10/N1T (Casaerale et al., Virology 156:40-49, 1987), (-): control bacterial lysate.

Figure 4 shows a Western blot analysis of cleavage products derived from the Gag-PR polyproteins co-expressed with Vif proteins in bacterial cells. Blots were stained with antisera to CA, MA, PR, and Vif. UN: uninduced bacterial assay; 1 hour, 2 hour: time post-induction by IPTG.

Figure 5 shows a Western blot of bacterial extracts prepared from cells expressing Gag-PR and CA-PR with or without mutations at the cleavage sites between $p6^{Pol}$ and PR. The polyproteins were co-expressed with pGST or pGST-

Vif. The blot was reacted with monoclonal anti-CA. The presumptive processed polypeptides are indicated at the right.

Figure 6 shows a Western blot of bacterial cells carrying a $p6^{Pol}$-PR encoding plasmid (Almog et al., J.Virol. 70:7228-7232, 1996) co-expressed with plasmids encoding either GST-Tat (T), GST-ΔVif (ΔV), GST-C'Vif (C'V), GST-N-Vif, (N'V), GST (G), or GST-Vif (V) using antiserum to PR. The extracts were prepared 30 min after induction. UN: uninduced. The presumptive processed polypeptides are indicated at the right.

Figure 7 shows a kinetic analysis of autoproteolysis of a minimal substrate. Panel A shows a Western blot as described in Figure 6, extracts prepared at 30 minute intervals. Panel B shows a densitometric analysis of Western blot in Figure 7A comparing the fraction of signal in PR versus the total signal in PR with $p6^{Pol}$-PR for each time point. Panel C shows a schematic diagram of the Vif regions relative to inhibition of proteolysis: the black bar indicates the GST protein fused to the N'terminus of each Vif construct, the open bar indicates the region of Vif which is expressed relative to the intact 192 amino acid protein, the dashed line indicates regions from the N terminus or the internal section of Vif deleted from the corresponding expression vector. Western blot analysis is summarized in the column to the right.

Figure 8. Panel A shows an in vitro cleavage assay of a chromophoric synthetic peptide substrate (S) by purified HIV-1 protease (P) in the presence or absence of the protease inhibitor Ro 31-8959 (PI) (10 μM) as determined by a decrease in absorbance at 310 nm (A_{310}). Panel B shows an in vitro assay assessing effect of Vif on substrate cleavage by protease using purified, full length Vif, GST-N'Vif, purified Tat or GST-C'Vif. C and D show a dose response of GST-N'Vif (C) and GST-C'Vif (D) on protease-mediated cleavage of substrate.

Figure 9. Panel A shows PR immobilized on Protein A agarose beads bearing rabbit anti-PR. GST-follistatin, GST-Vif, GST-N'Vif, or GST-C'Vif were mixed with beads, analysed in Western blot by staining with anti-GST. Purified

GSTN'Vif was loaded in one lane as a positive control. Panel B shows a repeat of the same experiment testing binding of GST-N'Vif.

Figure 10. Panel A shows a sequence of peptides derived from the N'-terminus of the HIV-1 Vif protein that were synthesized and tested in this study. Panels B and C show peptides that were screened for PR inhibition at a concentration of 1mM (panel B) or at increasing concentrations (panel C) in standard reaction mixtures of 10µl. Roche RO 31-8959 (0.1mM) and Pepstatin A (0.2mM) were used as controls for PR activity. Panel D shows pepsin studies.

Figure 11. Panel A shows Vif-derived peptides immobilized on nitrocellulose filters and exposed to $p6^{Pol}$-PR, PR, or mutated PR . Binding of PR was detected by anti-PR and fluorography. Panel B shows the same experiments under different conditions of binding. Spots 2 and 3 in the last row contain GST-Vif or RSV peptide, respectively, as indicated.

Figure 12. Panel A shows an analysis of binding of PR to Vif-derived peptides on wells by ELISA. The upper panel shows results of binding to peptide coated wells by PR, the lower panel shows results of preincubation of PR with the indicated peptide prior to addition of the mixture to peptide coated wells. Panel B shows binding of purified PR to Vif-derived peptides, as determined by ELISA. Binding of PR to the Vif-derived peptides attached to the wells, competitive binding experiments, or preincubation with 14 nM of RO 31-5989. Panel C shows dose-dependent binding of PR to Vif-derived peptides. The assay was carried as described in panel B with increasing concentrations of PR. Panel D shows competitive inhibition of PR binding by Vif-derived peptides. PR (50 ng) was preincubated for 18 hr in 200 µl with increasing concentrations of cognate peptide before adding the mixture to the peptide-coated microwells (without addition of peptide, 1µg/ml, 10 µg/ml, and 50 µg/ml of the cognate peptides). Panel E shows the binding of PR to short peptides in an ELISA assay; closed bars indicate binding to peptide-coated wells by PR, open bars show the results of preincubation of PR with indicated peptide.

Figure 13 shows the inhibition of PR activity and virion maturation by Vif peptides in an eucaryotic expression system using cells infected with the vaccinia vector expressing HIV-1 Gag-Pol, vVK-1, and cultured in the presence of the indicated Vif-derived peptide. Cells and extracellular virus-like particles were harvested and analysed by Western blot for Gag proteins using monoclonal anti-CA antibody. Panels A and B show Gag expression in Hut-78 cells and their particles, Panels C and D show the same analysis using vVK-1 infected CEM cells, and Panels E and F show the same analysis using vVK-1 infected PBL. The lane labelled pT5 contains a bacterial lysate from cells expressing the HIV-1 construct covering matrix through PR. The mobility of $p55^{Gag}$, $p41^{Gag}$, and $p24^{Gag}$ are indicated at the right of each autoradiogram.

Figure 14 shows inhibition of HIV-1 maturation by Vif-derived peptides. Hut 78 Cells (Panel A) and extracellular virions (Panel B) were harvested and analyzed by Western blotting using monoclonal anti-CA antibody.

Figure 15 shows reduction of virus production followed by treatment with Vif derived peptides in chronically infected Hut 78 cells (Panel A), and newly infected cells (Panel B).

Figure 16. Panel A shows reduction of HIV-1 production in PBL after exposure to Vif-derived peptides. Panel B shows dose-dependent effects of a Vif-derived peptide on HIV-1 production in PBL.

Detailed Description of the Invention

The present invention is directed to novel inhibitors of an HIV or other lentiviral or retroviral protease which are capable of inhibiting HIV or other lentiviral or retroviral replication. As such, the inhibitors are capable of reducing, eliminating or preventing viral infection. The inhibitors are peptides and polypeptides having an amino acid sequence which corresponds to the amino acid sequence of a lentiviral Vif protein (Vif-derived protease inhibitor). The invention is also directed to compositions containing the inhibitors of the invention and to

methods for using such compositions in the prevention or treatment of HIV or other lentiviral and retroviral infections. The invention is also directed to methods for identifying such inhibitors in cellular model systems, HIV-infected cells, in vitro high flux assay systems, and animal model systems.

The Vif-derived protease inhibitors are derived from the amino acid sequence of a lentiviral Vif protein., and are capable of inhibiting the function of a lentiviral or retroviral protease. Such peptides and polypeptides are therefore comprised of amino acid sequences which are the same as, substantially correspond to, or are analogous or homologous to the sequences from the lentiviral Vif protein. The peptide inhibitors are preferably derived from the HIV-1 Vif protein which is a protein that modulates the activity of the HIV-1 protease. The Vif-derived protease inhibitors are defined functionally by their ability to inhibit the proteolytic activity of an HIV or lentiviral or retroviral protease, i.e., reduce or eliminate the ability of the enzyme to cleave a native or synthetic substrate. Analogs, homologs, derivatives, truncated fragments and chimeras of the Vif-derived protease inhibitors which retain this functional property are within the scope of the invention.

As used herein, the term "peptide" refers to an oligomer of at least two contiguous amino acids, linked together by a peptide bond., and not greater than fifty amino acids. As used herein, the term “polypeptide” refers to an oligomer of at least fifty amino acids.

As used herein, "substantially corresponds" means an inhibitor amino acid sequence having approximately 70% identity in amino acid sequence to a Vif-derived peptide or polypeptide, whether colinear or including gaps in the parent sequence, and which retain the functional capability of the parent peptide. Methods for characterizing identity relationships among two or more amino acid sequences can include the use of algorithms (e.g., as decribed in Molecular Sequence Comparison and Alignment, in Nucleic Acid and Protein Sequence Analysis, Bishop, M. et al., eds., IRL Press, Oxford 1987).

By "homolog" is meant the corresponding peptides or polypeptides from other Vif or Vif-like proteins from other HIV strains, lentiviruses, or retroviruses, so long as the structural and functional properties of the peptides are retained.

By "analog" is meant substitutions, rearrangements, deletions, truncations and additions in the amino acid sequence of a Vif-derived protease inhibitor, so long as the structural and functional properties of the inhibitors are retained. Analogs also include inhibitors which contain additional amino acids added to either end of the peptides that do not affect biological activity, e.g., the presence of inert sequences added to a functional inhibitor which are added to prevent degradation. In another embodiment, conservative amino acid substitutions can be introduced into an inhibitor provided that the functional activity of the inhibitor is retained.

The criticality of particular amino acid residues in an inhibitor may be tested by altering or replacing the residue of interest. For example, the requirement for a cysteine residue, which can be involved in the formation of intramolecular or intermolecular disulfide bonds, can be tested by mutagenesis of the cysteine to another amino acid, for example, tyrosine, which cannot form such a bond.

The Vif or Vif-like protein which serves as the parent amino acid sequence for the inhibitors of the invention can be derived from any lentivirus or other retrovirus, including, but not limited to, HIV-1, HIV-2, simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), bovine immunodeficiency virus (BIV), visna virus and all isolates thereof.

The sequence of an inhibitor of the invention can include all or part of the amino acid sequence of the HIV-1 Vif protein, which contains 192 amino acids. A preferred length for an inhibitor of the invention ranges from 4 amino acids to 35 amino acids.

Inhibitors of the invention are described with reference to the following amino acid nomenclature wherein

A = Ala = Alanine

R = Arg = Arginine

N = Asn = Asparagine

D = Asp = Aspartic acid

B = Asx = Asparagine or aspartic acid

C = Cys = Cysteine

Q = Gln = Glutamine

E = Glu = Glutamic acid

Z = Glx = Glutamic or glutamic acid

G = Gly = Glycine

H = His = Histidine

I = Ile = Isoleucine

L = Leu = Leucine

K = Lys = Lysine

M = Met = Methionine

F = Phe = Phenylalanine

P = Pro = Proline

S = Ser = Serine

T = Thr = Threonine

W = Trp = Tryptophan

Y = Tyr = Tyrosine

V = Val = Valine

The inhibitors of the invention are preferably derived from the N-terminal half of the Vif protein (N'Vif, amino acids 1-96), which has been shown to inhibit HIV-1 protease-mediated proteolysis. The peptides may also be derived from the C-terminal half of the Vif protein (C'Vif, amino acids 97-192), which has been shown to mediate the membrane association of the Vif protein (Goncalves et al., J. Virol. 69:7196-7204, 1995).

In preferred embodiments, specific peptide inhibitors of the invention are the following peptides which have an amino acid sequence derived from the HIV-1

Vif protein (numbering and sequence derived from strain HIV-1/N1T-A (Sakai et al., J. Virol. 65:5765-5773, 1991) or closely related HIV-1 BH10 (Ratner et al., Nature 313:277-284, 1985; SEQ ID NOs: 15-20).

MENRWQVM (1-8) (SEQ ID NO: 1)

IVWQVDRM (9-16) (SEQ ID NO: 2)

RIRTWKSLVKHHM (17-29) (SEQ ID NO: 3)

YVSGKARGWFYRHHYESPHPRISSEVHIPLGDARLV (30-65) (SEQ ID NO: 4)

YVSGKARGWFYRHHY (30-44) (SEQ ID NO: 5)

ISSEVHIPLGDARLV (51-65) (SEQ ID NO: 6)

YVSGKARG (30-37) (SEQ ID NO: 7)

GKARGWFY (33-40) (SEQ ID NO: 8)

GWFYRHHY (37-44) (SEQ ID NO: 9)

ISSEVHIPL (51-59) (SEQ ID NO: 10)

VHIPLGDA (55-62) (SEQ ID NO: 11)

PLGDARLV (58-65) (SEQ ID NO: 12)

LGDARLVITTYWGLHT (59-74) (SEQ ID NO: 13)

ITTYWGLHTGERDWHL (66-81) (SEQ ID NO: 14)

DWHLGQGVSIEWRKK (78-92) (SEQ ID NO: 15)

DWHLGQGV (78-85) (SEQ ID NO: 16)

LGQGVSIE (81-88) (SEQ ID NO: 17)

VSIEWRKK (85-92) (SEQ ID NO: 18)

EWRKKRYSTQV (88-98) (SEQ ID NO: 19)

EWRKKRYS (88-95) (SEQ ID NO: 20)

YVSGARGWFYRHHYE (30-45) (SEQ ID NO: 21)

RHHYESPHPRISSEV (41-55) (SEQ ID NO: 22)

DARVITTYWGLHTG (61-75) (SEQ ID NO: 23)

ITTYWGLHTGERDWH (65-80) (SEQ ID NO: 24)

LGQGVSIKWRKKRYS (81-95) (SEQ ID NO: 25)

MENRWQVMIVWQVDRM (1-16) (SEQ ID NO: 26)

WKSLVKHHMYVSGKARGWFYRHHYESPHPRISSEVHIPLGDARLV (21-65) (SEQ ID NO: 42)

RHHYESPHPRISSEVHIPLGDARLV (41-65) (SEQ ID NO: 43)

The following peptides represent consensus sequences which are derived from Vif sequences contained in the Los Alamos database (Myers et al., eds., Human Retroviruses and AIDS, Los Alamos, New Mexico, 1993):

WHSLIKYLKYKTKDL (23-38) (SEQ ID NO: 27)

YVSKKARGWFYRHHYE (30-45) (SEQ ID NO: 28)

RHHYESTNPRISSEV (41-55) (SEQ ID NO: 29)

YFDCFEDSAIRKALLGHIVSPRC (111-133) (SEQ ID NO: 30)

SLQYLALAALI (144-154) (SEQ ID NO: 31)

Peptide inhibitors of the invention also include, inter alia, the following peptides containing consensus sequences which correspond to the major conserved domains of Vif (Myers et al., eds., Human Retroviruses and AIDS, Los Alamos, New Mexico):

^{1}MENRXXVMIVWQXDRM (SEQ ID NO: 32)

^{40}YXHHY (SEQ ID NO: 33)

^{70}TYWGL (SEQ ID NO: 34)

^{101}DPXLAD (SEQ ID NO: 35)

^{110}YFDCF (SEQ ID NO: 36)

^{147}LQYLALXXP (SEQ ID NO: 37)

^{163}PPLPXVXKLTEDRWNKP (SEQ ID NO: 38)

Chimeric protease inhibitors which combine one or more of the preferred peptide inhibitors or segments thereof or fragments of polypeptide inhibitors are within the scope of the invention.

Protease inhibitors of the present invention also include cyclic or derivatized peptides, and further include peptides containing D-amino acids as well as L-amino acids. Inhibitors which retain the functional properties of the Vif-

derived protease inhibitors and are derived by techniques of combinatorial chemistry known to those skilled in the art are within the scope of the invention.

The peptide and polypeptide inhibitors of the invention can be synthesized according to Merrifield solid-phase synthesis techniques (Kotler et al., Proc. Natl. Acad. Sci. 85:4185-4189, 1985; Barany et al., in Gross et al., eds., The Peptides, Vol. 2, Academic Press, 1980) or other techniques of peptide synthesis known to those skilled in the art. After cleavage and deprotection, synthetic peptides can be purified by, for example, gel filtration chromatography and any reverse-phase column/HPLC system known to those skilled in the art.

Peptides and polypeptides may also be prepared by standard recombinant DNA technology using techniques well known to those skilled in the art for nucleotide-based peptide design (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Press, Cold Spring Harbor, New York; Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, New York, 1995). Site-directed mutagenesis using recombinant DNA techniques, for example, may be used to prepare peptide analogs and homologs from parent peptides.

The amino acid sequences of the peptides and polypeptides can be confirmed and identified by amino acid composition analysis as well as manual and automated Edman degradation and determination of each amino acid, HPLC analysis, or mass spectrometry.

Polypeptide inhibitors derived from the Vif protein may also be produced by chemical or enzymatic digestion of the full-length protein using techniques that are known to those skilled in the art.

The HIV protease which is used in assays to detect inhibitors of the invention may be recovered by purification from viral particles, synthesized in full, or may be produced by recombinant DNA technology using techniques known to those skilled in the art.

The N-terminal amino acid of the peptides may contain a free amino group or be acetylated, and the C-terminal amino acid of the peptide may be amidated or comprise a free carboxy group. Other modifications of the peptide termini known to those skilled in the art are within the scope of the invention.

The invention is further directed to providing assays to identify protease peptide inhibitors. Inhibitors are characterized by an ability to block the activity of protease in an active infection in vivo, a cellular model system, or in vitro assays. The effects of an inhibitor may also be characterized by its effects in altering, reducing or eliminating viral morphogenesis or replication, or virion infectivity.

As noted above, the native substrates for the HIV-1 protease are the $Pr55^{Gag}$ and $Pr160^{Gag\text{-}Pol}$ polyproteins which are processed to yield the core proteins p17 matrix (MA), p24 capsid (CA), nucleocapsid (NC), $p6^{Gag}$, and two spacer peptides, p2 and p1 from the Gag gene. In addition to structural proteins, $Pr160^{Gag\text{-}Pol}$ also comprises the viral protease (PR), reverse transcriptase, and integrase enzymes encoded by the Pol gene. Substrates for use in identifying the peptide inhibitors of the invention include these native polyprotein substrates, $Pr55^{Gag}$ and $Pr160^{Gag\text{-}Pol}$, substrates derived from these native substrates as truncated fragments containing one or more protease cleavage sites; and synthetic peptides encompassing all or part of the Gag and/or all of part of the Gag-Pol scissile (cleavable) sites. Also included are other lentiviral and retroviral Gag and Gag-Pol proteins which serve as substrates for their proteases.

Preferred substrates include HIV-1 Gag-PR, which includes a Gag-Pol fragment that contains PR and contains at least six cleavage sites for the protease; CA-PR, which includes truncated Gag polyprotein beginning at capsid p24 (CA) and a Pol fragment that includes PR; and $p6^{Pol}$-PR, containing only a segment of Pol with $p6^{Pol}$ and PR. This latter substrate contains only one major protease cleavage site and allows for a simple determination of protease activity by assaying for generation of the 11 kDa protease upon a processing event. Shorter length substrates may be designed to contain a specific protease cleavage site, e.g., the site

between Gag proteins matrix p17 and capsid p24, and the cleavage site between Pol proteins reverse transcriptase and integrase.

Substrates can be further designed to contain a defined subset of cleavage sites by site-specific mutagenesis of selected sites contained in the protein fragment. For example, the substrate Gag-PR can be modified to alter the cleavage site between $p6^{Pol}$ and PR, to prevent the release of PR from the polyprotein. Studies have shown that truncated polyproteins containing the $p6^{pol}$-PR fusion undergo efficient autoprocessing mediated by the PR. Thus, the release of PR from its precursor is not a prerequisite for PR activity (Kotler et al., J. Virol. 66:6781-6783, 1992; Almog et al., J. Virol. 70:7228-7232, 1996; Zybarth et al., J. Virol. 68:240-250, 1994).

With a substrate having known protease cleavage sites, the molecular weights of the expected processed products can be predicted, as well as intermediate products generated by incomplete processing. Detection of the protein products resulting from an assay can be performed by standard techniques for protein detection, including Western blotting using anti-HIV-1 serum or monoclonal antibodies against specific proteins, immunoprecipitation, Coumassie blue staining or radiolabeling of SDS-PAGE protein gels, ELISA assays, as well as other methods known to those skilled in the art. The resolution of lower molecular weight species can be accomplished using polyacrylamide gel electrophoresis, including gradient gels. Quantitation of protein levels following electrophoretic resolution can be performed using densitometry.

In vivo assays for identifying a Vif-derived protease inhibitor can be conducted in art-recognized cellular model systems in order to simulate the environment of an HIV-1 infection. Such assays can be performed in, for example, bacteria, yeast, insect cells, or mammalian cell culture. The activity of a Vif-derived protease inhibitor can be assayed by its effect on viral protease activity in the cell-based system. This can be accomplished by the introduction of the genes encoding protease and the test substrates on separate plasmids into a cellular model

system. Where the substrate itself includes the protease and is subject to autoproteolysis, no exogenous protease need be provided. Where the substrate does not include protease, the gene encoding protease also must be provided in the assay. A nucleic acid encoding a candidate Vif-derived protease inhibitor can also be engineered into the PR-containing plasmid downstream from the PR gene, using an oligonucleotide encoding the inhibitor and appropriate regulatory elements that ensure transcription and translation along with the PR gene. Alternatively, a Vif-derived protease inhibitor can be provided exogenously in the growth medium. Analysis of the processed protein products produced in the presence of a Vif-derived protease inhibitor reveals the extent of the inhibition of protease activity.

Alternatively, the cells used in the assay can be engineered to constitutively or inducibly express an autoproteolyzable substrate (i.e., containing protease), by stable transfection of a DNA sequence encoding such a substrate. Such cells can be transfected with a Vif-derived protease inhibitor which is expressed in the cell, or supplied with a Vif-derived protease inhibitor in the growth medium and the nature of the processed protein products can be determined. This assay can be accomplished using one plasmid that is engineered to contain protease and a Vif-derived protease inhibitor. Where a substrate does include protease, such a plasmid need only provide the DNA sequence encoding a Vif-derived protease inhibitor.

The cellular model assays can further be performed using a three-plasmid system where protease, substrate and a Vif-derived protease inhibitor are encoded on separate plasmids, each preferably containing different selectable markers to allow for the selection of cells transformed with all plasmids.

In a particular embodiment of an in vivo assay, a bacterial two-plasmid expression system is used in which one plasmid carries DNA encoding a Vif-derived protease inhibitor and another plasmid is engineered to carry an HIV-1 Gag-Pol gene fragment that encodes a protein having protease-dependent cleavage sites. The Gag-Pol fragment provides for production of the protease and also

provides a protein substrate susceptible to cleavage by the protease in an autoproteolytic manner. In this system, protease is equimolar to substrate. Both plasmids transform a suitable bacterial host such as an *E. coli* BL21 (DE3), where expression from the plasmids is controlled by using an inducible promoter in the plasmids or an inducible RNA polymerase. Preferably, the bacterial host cell is engineered to contain an RNA polymerase, such as the T7 polymerase, that is under the control of an inducible promoter. The coding sequences in each plasmid are each under the regulatory control of a promoter that is responsive to the inducible polymerase engineered into the host.

In a specific preferred embodiment of the bacterial assay, the coding sequences for a Vif -derived protease inhibitor and a Gag-Pol substrate are engineered into a separate pGEX vector and a separate pT5 vector, respectively, (Pharmacia, Piscataway, NJ). An E. coli BL21 (DE3) host cell carrying the T7 polymerase gene under the control of the lacUV5 promoter is transformed by both plasmids. Preferably, a nucleic acid encoding the specific substrate Gag-PR is used, which spans the Gag gene and contains the Pol gene up to the protease coding region and has a molecular weight of 68 kDa. A frameshift mutation is introduced at the Gag-Pol junction to permit translation in a single reading frame. This substrate has five or six protease cleavage sites. The mature proteins can be predicted as well as all combinatorial possibilities from intermediates in the processing events (see Fig. 3). The single polyprotein includes MA, CA, p2, NC, $p6^{Pol}$ and PR. IPTG is used to induce the lacUV5-dependent expression of the T7 polymerase. The polymerase, in turn, generates expression of the genes under the control of the T7 promoter. The results of any cleavage of the protein substrate is observed by recovering the proteins in a cell lysate and analyzing the proteolytic processing events by, for example, a Western blot using anti-HIV antibodies or other standard techniques of protein identification known to those skilled in the art.

Other plasmids which could be used in such assays, include, but are not limited to, PQE (Qiagen, Chatsworth, CA), pET (Navagene, Madison, WI) as well

as any plasmid which supports expression of the heterologous DNA sequences. Preferably, the plasmids used in the bacterial expression assays also contain regulatory sequences permitting expression in eucaryotic cells, so that inhibitors identified in a procaryotic assay system can be tested in a eucaryotic assay system.

Eucaryotic cellular model systems which can be used to assay the inhibitors of the invention simulate the natural environment of the viral infection to which the inhibitors are directed. An assay system which employs a vector-delivered full or partial HIV genome into a eucaryotic cell can be used to simulate the production of viral proteins and virion production.

In a preferred embodiment, the vaccinia virus vector vVK-1, containing the Gag-Pol DNA fragment is used to infect suitable host cells. vVK-1 infection of many human cell types, including primary T lymphocytes, results in high levels of Gag-Pol production within 24h of infection and also permits the native PR-mediated processing of Gag-Pol and the assembly of virion-like particles which can be isolated from the cell supernatants (Karacostas et al., Proc. Natl. Acad. Sci. USA 86:8964-8967, 1989). Infection of the cells can be conducted in medium containing an inhibitor of the invention, and the effects on proteolytic processing and virion production can be determined. Protein analysis can be performed with standard techniques known to those skilled in the art. Virion-like particle production can assayed by recovering viral-like particles from cell supernatants, and determining their structural or compositional properties. Cells which can be used in these assays include macrophages, peripheral blood lymphocytes (PBL), including T cells, or established cell lines such as the known T-cell leukemia cell lines Hut-78, CEM, H4/CD4, and H9. Other eucaryotic cellular model systems include any plasmid or viral vector-based DNA delivery system which can be used to transfer the DNA for any combination of protease, substrate, inhibitor and/or viral genome that is necessary for an assay of virion production and/or infectivity. An example of such a delivery system which can be used in the invention is the

pIND/pVgRXR inducible plasmid eucaryotic expression system (Invitrogen, Carlsbad, CA).

Assays for Vif-derived protease inhibitors can also be performed in HIV-1 infected cells. Vif-derived protease inhibitors can be identified by their ability to inhibit or reduce HIV infection in eucaryotic cells, with or without detectable alteration in virion or intracellular virus protein structure or composition. Where the inhibitor is incubated with HIV infected cells, the production of infectious virus progeny is determined relative to control experiments without inhibitor.

Specific HIV strains which can be used in the assays of the invention include HIV/ADA, HIV-1_{IIIB}, CR10/N1T-A, NL4-3, NDK, and others.

The cells in such assays are infected with HIV or other lentiviral or other retroviral virions or transfected with proviral DNA comprising a desired virus genome. The Vif-derived protease inhibitor is added to the culture medium, or, alternatively, the DNA therefor is contained in an expression plasmid which is transfected into the virally-infected cell.

To assess whether a Vif-derived protease inhibitor reduces or eliminates the infectious process, the level and type of infectious progeny are assayed at suitable times post-infection. Evidence of microscopically observed viral spread, cytopathic effect, and increased amounts of the p24 capsid protein can provide an assessment as to whether infectious progeny are being generated. The assessment of progeny virus infectivity may be determined further by recovery of infected cells and co-cultivation with suitable cells (e.g., PBL or macrophages) or by the recovery of supernatant from the infected cells and cell-free infection of suitable cells. Suitable cells are defined as cells which require Vif for infection (e.g., PBL and macrophages but not Sup T1).

Progeny virus with aberrant morphology may be observed, e.g., by electron microscopy, in the presence of a Vif-derived protease inhibitor. Analysis of the protein composition of progeny virus produced in the presence of Vif-derived protease inhibitors shows alteration in the normal composition of viral proteins,

e.g., altering the wild-type protein profile. For example, the mature proteins found in normal virus particles may be absent or reduced (e.g., capsid p24, matrix p17). Also, increased levels of higher molecular weight species (e.g., Gag-specific p55, p41 and p38) may be observed, which indicate incomplete polyprotein processing, and therefore the presence of a functional Vif-derived protease inhibitor.

Quantitative assessment of an HIV-1 infection conducted in the presence of a Vif-derived protease inhibitor can also be determined using molecular markers, for example, by assaying viral p24 production by ELISA assay, reverse transcriptase activity, or viral DNA synthesis by quantitative PCR using standard techniques known to those skilled in the art.

Primary cells or cell lines which can be used for Vif-derived protease inhibitor studies in the context of an HIV-1 infection are preferably those that are susceptible to such viral infection. Such cells include, for example, PBL, macrophages, and the H4/CD4 and H9 cell lines.

Preferably, H4/CD4 cells, a human glioma cell line engineered to express the cell surface protein CD4, is used for assays of HIV-1 infection in the presence of a Vif-derived protease inhibitor in eucaryotic cells. H4/CD4 cells are highly susceptible to infection with HIV-1, both by standard infection methods and by transfection with proviral DNA. Efficient transfectability of these cells permits the evaluation of candidate Vif-derived protease inhibitors which have shown protease-inhibiting activity in bacteria, eucaryotic or in vitro assays.

The Vif-derived protease inhibitors of the invention can also be identified and characterized using in vitro assays. Cell-free assays using protease and native or synthetic substrates can be used to identify Vif-derived protease inhibitors which exert their effect by altering, reducing, or inhibiting the effect of Vif on protease activity. In such assays, the inhibitor is added to the combination of protease and substrate, and the effect of the inhibitor on protease-mediated cleavage of one or more substrates is determined.

Substrates for such assays include any protein or peptide which contains one or more of the HIV-1 protease cleavage sites, including those described infra. Because the molecular weights of the processed products can be predicted, deviation from the expected proteolytic products indicates that a Vif-derived protease inhibitor is capable of altering, reducing or inhibiting protease activity. Any techniques for protein identification can be used in these assays to detect the proteolytic products, including Western blots using antiserum to the mature proteins, immunoprecipitation and Coumassie blue-stained SDS-PAGE analysis.

In vitro assays can also be performed in high flux evaluation in order to screen large numbers of candidate inhibitors. Specific substrates can be designed for use in a high flux assay system, where cleavage is monitored by a signal system amenable to ready detection. For example, a protease substrate can include a chromophoric tag which is detectable by spectrophometry, and which changes its absorption properties upon substrate hydrolysis, i.e., hydrolysis produces a detectable chromophore. A fluorogenic substrate can be designed for detection by fluorescent emission by linking a fluorophore to a residue in the substrate and linking a chromophore to a nearby residue on the opposite side of a cleavage site. Upon cleavage of the substrate, the residues are separated and the chromophore no longer quenches the fluorescent emission, and such emission can be detected in a fluorometer. The use of other signal systems which facilitate high flux identification of candidate inhibitors is within the scope of the invention.

Minimal protease-susceptible substrates can be used for the in vitro or high flux assays. Such substrates include, for example, a chromophoric peptide encoding the cleavage site between reverse transcriptase and integrase: Acetyl-Arg-Lys-Ile-Leu'Phe(NO2)-Leu-Asp-GLy-NH2, where the peptide is cleaved between the Leu and Phe residues, and such cleavage can be detected by a decrease in spectrophotometric absorbance at 310 nm. Another substrate for an in vitro assay includes a chromogenic peptide encoding the cleavage site at the junction of $p6^{Pol}$ and protease in the Pol gene: Ser-Phe-Asn-Phe'Pro-Gln-Ile-Thr, where

nitrophenylalanine is substituted for the phenylalanine flanking the cleavage site. Cleavage by the protease is also detected by a decrease in spectrophotometric absorbance at 310 nm. Another substrate includes a peptide that contains the cleavage site between the p17 matrix (MA) and p24 capsid (CA) proteins, and comprises the sequence Arg-Gln-Ser-Gln-Asn-Tyr'Pro-Ile-Val-Lys-Arg, in which a fluorophore is linked to the glutamine residue and an acceptor chromophore is linked to the lysine residue. Fluorescence is quenched by the proximity of the chromophore; upon cleavage, the residues separate and the dequenched emission can be detected in a fluorometer.

In vitro assays also include binding studies which determine the physical interaction between protease and a Vif-derived protease inhibitor. Such assays are performed by incubating protease and an inhibitor and determining complex formation by, for example, Western blot of immunoprecipitated proteins. Alternatively, an inhibitor may be applied to nitrocellulose filters or microtiter well which are then incubated with protease, and binding can be detected in an ELISA format, e.g., incubation with anti-protease antiserum, followed by a secondary antiserum which contains a signal that is readily assayed, e.g., by cleavage of a chomophoric substrate.

The in vitro assays are performed by incubating protease, Vif-derived protease inhibitor and substrate, using a range of molar ratios between inhibitor and protease in order to assess the degree of inhibition by inhibitor on protease activity. Determination of the kinetic parameters of protease-catalyzed proteolysis such as Km and Kcat of cleavage sites is carried out by using increasing concentrations of a specific substrate, e.g., a chromophoric substrate. The inhibition constant (Ki) can be determined under identical conditions using a fixed concentration of substrate (Tomaszak et al., Biochem. Biophys. Res. Comm. 168:274, 1980).

The reagents for the in vitro assays can be synthesized using peptide synthetic techniques or recombinant DNA technology, described infra. Where

recombinant DNA techniques are used to produce a peptide or protein reagent for these assays, they may be produced as fusion products which permit ready recovery and purification. For example, the gene for an HIV protease can be engineered into an expression plasmid and transformed into bacterial cells such that it is expressed as a fusion protein containing a molecular tag such as glutathione-S-transferase (GST) or 6 histidine residues. The tag allows purification of the expressed protein using beads or columns coated with glutathione to purify GST-tagged proteins or coated with nickel to purify histidine-tagged proteins. Purified protease obtained by standard biochemical purification techniques from viral particles can also be used.

Candidate inhibitors of the invention can also be obtained using peptide libraries which contain large numbers of peptides for screening.

An oligonucleotide endoding a Vif-derived protease inhibitor can be used to engineer cell lines which constitutively express the inhibitor in order to test the effect of an inhibitor on different isolates of HIV-1 or other HIV strains. Such isolates include lymphotropic and macrophage-tropic strains, primary strains derived from blood cells or tissues, and North American, European, African and Asian isolates.

The peptide inhibitors of the invention can be tested in animal models of HIV infection, including the SCID-Hu mouse model of HIV-1 infection (Aldrovandi et al., J.Virol. 70:1505, 1996) and SIV-infected monkeys. Such models of infection are suitable for testing the inhibitors of the invention for efficacy against challenge with HIV or other lentiviruses and other retroviruses in order to identify those inhibitors which can be used for prevention or treatment of viral infection.

The inhibitors of the invention can be assayed to determine the concentration required to achieve an antiviral effect against a target virus. A convenient variable for measurement is the concentration of an inhibitor required to inhibit 50% of viral replication (IC_{50}), whether assayed in cell culture or with the

use of a molecular marker such as the measurement of viral p24 production by ELISA assay, presence of viral RNA, reverse transcriptase activity, or viral DNA synthesis by quantitative PCR using standard techniques known to those skilled in the art.

Peptides of the invention can be evaluated for cytotoxic effects using standard assays that measure cell viability. Such assays include ^{14}C protein hydrolysate, ^{3}H thymidine uptake, MTT reduction, and cell growth. Such parameters as TD_{50} (toxic dose to 50% of the tested culture) can be derived from such assays. Comparison of the TD_{50} with the IC_{50} (inhibitor concentration required to inhibit 50% of the viral marker being tested or viral replication) can indicate a therapeutic index for a particular compound (TI). Preferably, a prospective inhibitor of the invention has an IC_{50} that is at least ten times higher than the TD_{50}, and the IC_{50} is effective at a minimum concentration of 10^{-6} M in culture. Most preferably, an inhibitor of the invention exhibits an IC_{50} of 10^{-7} M or 10^{-8} M.

The inhibitors of the invention are useful in the isolation of HIV or other lentiviral and retroviral protease mutants which can be used in subsequent screens to identify other antiviral agents to which they are susceptible. The inhibitors are also useful in the three-dimensional analysis of the Vif-protease interaction.

The inhibitors of the invention can also be used to inhibit any viral protein in addition to or instead of a viral protease, including the Vif protein, in order to achieve an antiviral effect which is useful in the prevention or treatment of HIV and other lentiviral and retroviral infections.

In view of the above noted properties of the inhibitors of the invention, it is further contemplated that these inhibitors may be used in compositions for the prevention or treatment of an HIV or other lentiviral and retroviral infections, and the treatment of consequent pathologic conditions such as AIDS. Another aspect of the invention is directed to methods for preventing and treating an HIV or other lentiviral or retroviral infection by administering a composition containing one or

more of the inhibitors of the invention to a patient for a time and under conditions to accomplish such result.

The inhibitors, compositions and methods of the invention can be used in the treatment of HIV-positive individuals, including those exhibiting the conditions of AIDS-related complex (ARC) and AIDS, as well as those who are asymtomatic. These inhibitors, compositions and methods can also be used in the prophylaxis of HIV or other lentiviral and retroviral infections, and can also be used the treatment or prophylaxis of veterinary infections caused by lentiviruses and other retroviruses.

The peptides of the invention may be used in combination with other inhibitors of HIV replication, including, but not limited to, other antiviral compounds, immunomodulators, antibiotics, vaccines, chemokines and other therapeutic agents. Particular agents which can be used in combination with the inhibitors of the invention include agents such as azidothymidine (AZT), dideoxyinosine (DDI), dideoxycytosine, saquinavir, indinavir, ritonavir, (DDC), and other antiviral compounds. The inhibitors of the invention may also be used in combination with agents which are used to treat secondary complications of HIV infection, e.g., gancyclovir used in the treatment of cytomegalovirus retinitis. Combination therapy may retard the development of drug-resistant mutants by requiring multiple mutation events for the emergence of a fully drug-resistant isolate.

The inhibitors of the present invention may be administered to a host as a composition in an amount effective to inhibit viral replication and/or infection, together with a physiologically acceptable carrier.

The inhibitors of the invention may be administered systemically for preventing or treating an HIV or other lentiviral or retroviral infection. When used systemically, the inhibitor compositions may be formulated as liquids, pills, tablets, lozenges or the like, for enteral administration, or in liquid form for parenteral injection. The peptides and/or polypeptides (or inhibitor-protein

conjugates) may be combined with other ingredients such as carriers and/or adjuvants. There are no limitations on the nature of such other ingredients, except that they must be physiologically acceptable, efficacious for their intended administration and cannot degrade the activity of the active ingredients of the compositions. An inhibitor can also be covalently attached to a protein carrier, such as albumin, so as to minimize diffusion of the inhibitor.

As used herein, physiologically acceptable carriers include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic agents and the like. The use of such media and agents are well-known in the art.

The forms of the compositions suitable for injection include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the ultimate solution form must be sterile and fluid. Typical carriers include a solvent or dispersion medium containing, for example, water buffered aqueous solutions (i.e., biocompatible buffers), ethanol, polyol such as glycerol, propylene glycol, polyethylene glycol, suitable mixtures thereof, surfactants or vegetable oils. Sterilization can be accomplished by an art-recognized technique, including but not limited to, filtration or addition of antibacterial or antifungal agents, for example, paraben, chlorobutanol, phenol, sorbic acid or thimerosal. Further, isotonic agents such as sugars or sodium chloride may be incorporated in the subject compositions.

Production of sterile injectable solutions containing the subject inhibitors is accomplished by incorporating these compounds in the required amount in the appropriate solvent with various ingredients enumerated above, as required, followed by sterilization, preferably filter sterilization. To obtain a sterile powder, the above solutions are vacuum-dried or freeze-dried as necessary.

The peptide compositions also may be impregnated into transdermal patches, plasters and bandages, preferably in a liquid or semi-liquid form.

When the inhibitors of the invention are administered orally, the compositions thereof containing an effective dosage of the peptide may also

contain an inert diluent, an assimilable edible carrier and the like, be in hard or soft shell gelatin capsules, be compressed into tablets, or may be in an elixir, suspension, syrup or the like.

The subject inhibitors are thus compounded for convenient and effective administration in an amount effective to inhibit viral replication and/or infection, together with a physiologically acceptable carrier.

The precise therapeutically effective amount of inhibitor to be used in the methods of this invention to prevent or treat an HIV infection cannot be stated because of the nature of the infectious process. It must be noted that the amount of inhibitor to be administered will vary with the degree of infection in an individual, as determined by such parameters as viral load and CD4 cell counts. Individual-specific variables such as age, weight, general health, gender, diet, and intake of other pharmaceuticals factor into the dosage range. The design of an optimal protocol for an infected individual may further consider the identity of the viral isolate(s) isolated from an infected individual with an infection for optimal result. A further consideration in protocol design would be the presence of a viral strain which is resistant to existing protease inhibitors or RT inhibitors.

The amount of an inhibitor of the invention per unit volume of combined medication for administration is also very difficult to specify, because it depends upon the amount of active ingredients that are afforded directly to the site of infection. However, it can generally be stated that a peptide or polypeptide inhibitor of the invention should preferably be present in an amount of at least about 1.0 nanogram per milliliter of combined composition, more preferably in an amount up to about 1.0 milligram per milliliter.

Systemic dosages depend on the age, weight and condition of the individual and on the administration route. For example, a suitable dosage for the administration to adult humans ranges from about 0.01 to about 100 mg per kilogram body weight. The preferred dosage ranges from about 0.5 to about 5.0 mg per kilogram body weight.

Since the peptide and polypeptide compositions of this invention are effective in reducing or eliminating the ability of HIV or other lentiviruses and other retroviruses to replicate and/or generate infectious progeny, a continual application or periodic reapplication of the compositions is indicated and preferred.

The peptide and polypeptide inhibitors of the invention can also be delivered to an individual by gene transfer techniques. DNA coding for a Vif-derived protease inhibitor of the invention can be delivered to the cells of an individual in need of such an inhibitor by any method of gene transfer known to those skilled in the art, including, but not limited to, viral vectors, lipid-mediated delivery, transfection, electroporation, as well as other methods. Viral vectors which can be used to deliver such inhibitors include those derived from DNA and RNA viruses, including, but not limited to, adenovirus, herpesvirus, poxvirus, retrovirus, and adeno-associated virus. (See PCT publication No. WO 95/05851, published March 2, 1995, which is incorporated herein by reference).

Parameters which are used to monitor the effect of an inhibitor of the invention administered to an individual with an established HIV infection or administered to an individual for prophylaxis include the use of CD4 counts, plasma viral RNA concentration, viral phenotype, p24 antigen concentration, viral phenotype, level of anti-HIV antibodies as well as other markers of the clinical progression of an HIV infection known to those skilled in the art.

It will be recognized that the inhibitors and methods of the invention can be used in the treatment or prevention of any other lentiviral or retroviral infection, including, but not limited to, those resulting from HIV-1, HIV-2, simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), bovine immunodeficiency virus (BIV), visna virus and all strains and isolates thereof.

The following examples further illustrate the invention.

Example 1: Bacterial Assays of Vif Function Using A Two Plasmid System

Methods

Bacterial expression vectors. The Gag gene and a 5' segment of the Pol gene from HXB-2 encompassing $p6^{Pol}$ and PR were amplified by PCR essentially as described (Kotler et al., J. Virol. 66:6781-6783, 1992). Primer 11 ($5'^{789}$GGCCATGGGTGCGAGAGCGTCAGTATTAAGC-3') and primer 15 ($5'^{2558}$-GCTAATGGCAAAATTTAAAGTGCACCC-3') were used to amplify the region encompassing MA-PR to create substrate Gag-PR. To permit translation through the Gag-Pol junction, a frameshift mutation was at position 2088 by inserting a single base, A, thus placing Gag and Pol in the same reading frame using primer 2 ($5'^{2107}$GAAGGCCAGATCTTCCCTTAAAAAATTAGCCTGTCT-3') (Fig. 1).

A construct of substrate CA-PR was created by amplification using primer 1 ($5'^{1185}$-CCTATAGTGCAGAACATCCAGGGGCAAATGG-3') and primer 15. The amplified Gag-PR or MA-PR DNA was cloned through pUC12N (Norrander et al., J. Biotechnol. 2:157-175, 1985; Vieria et al., Gene 19:259-268, 1982) into a modified pT5 which expresses the foreign gene under the control of a T7 polymerase promoter and contains the ampicillin resistance gene (Studier et al., Meth. Enzym. 185:60-89 1990).

To express a histidine tagged substrate $p6^{Pol}$-PR, $p6^{Pol}$-PR was amplified using primer 4 ($5'^{2080}$AGGGAAGATCTGGCCTTCCTACAAGGG-3') and primer 7 ($5'$-^{2553}CTAAAAATTTAAAGTGCAACCAAT-3') and was cloned into pQE_{31} (Qiagen, Chatworth, Calif.) which encodes in frame both the leading six histidines and a termination codon. A cleavage site mutation between $p6^{Pol}$ and PR was generated by replacing the Phe codon, normally found at the carboxyl terminus of $p6^{Pol}$ (position 2249) with an Ile codon, abrogating cleavage using primers 4 and 5 ($5'$-^{2281}CGGCCGTTGCCAAAGAGTGATCTGAGGGATGTTAAA-3').

Construction of GST-Vif fusion proteins. The pGEX-2T plasmid (Pharmacia, Piscataway, NJ), which carries the GST gene under the control of the P_{tac} promoter, was modified by cloning the 1 kbp PstI fragment from $pKan^r$ into

PstI site in the ampicillin resistance gene, generating plasmid PGEX-T^{Kan} (Fig. 2). The pGEX-2T plasmid contains the kanamycin resistance gene (Kan^r) to permit co-selection of two different expression plasmids in the same bacterial cell. The full-length Vif gene or Vif gene segments were amplified from pKS242 plasmid (Sakai et al., J. Virol. 65:5765-5773, 1991) using Pfu polymerase (Stratagene, La Jolla, CA) and primers containing BamHI and EcoRI restriction endonuclease sites at 5' and 3' ends, respectively. vfgex5 (5'-TGATTAGGATCCATGGAAAACAGATGGCAGG-3') and vfgex3 (5'-ACACAATGAATGGACACTAGAATTCATTAGAGG-3') were used for amplification of full length Vif. N-terminal Vif was amplified using vfgex5 and vfgexl (5'-CACATAAGAATTCCCTGAACTAGCA-3') and C'terminal Vif was amplified using vfgex2 (5'-GGAAAAAGGGATCCAGGATGGAAG-3') and vfgex3. The amplified DNA was cloned into the corresponding sites in pGEX-2T^{Kan} for translation in frame with the GST gene. pGEX-2TTat (Rhim et al., AIDS 7: 1116-1121, 1994) was obtained from A. Andrew Rice through AIDS Research and Reference Reagent Program, Bethesda, MD. The sequences and residue coordinates for N'Vif, C'Vif and ΔVif are shown in Table 1.

TABLE 1

NAME	RESIDUES	SEQUENCE
N'Vif	1-96	MENRWQVMIVWQVDRMRIRTWKSLVKHHMYV SGKARGWFYRHHYESPHPRISSEVHIPLGDARLV ITTYWGLHTGERDWHLGQGVSIKWRKKRYST (SEQ ID NO: 39)
C'Vif	97-192	QVDPELADQLIHLYYFDCFSDSAIRKALLGHIVSP RCEYQAGHNKVGSLQYLALAALITPKKIKPPLPS VTKLTEDRWNKPQKTKGHRRSHTMNGH (SEQ ID NO: 40)
Δ-Vif		MENRWQVMIVWQVDRMRIRTWKSLVKHHMYV SGKARGWNKFRSTHPTRGC (SEQ ID NO: 41)

Bacterial transformation. E. coli BL21 (DE3) PlysS which carries an integrated T7 polymerase gene under the control of the lacUV5 promoter (Novagen, Madison, WI) were co-transformed with pGag-PR, $pGag_xPR$, PCA-PR, PCA_xPR, $p6^{Pol}$-PR, $p6^{Pol}{}_xPR$ and either pGEX-2T Vif, pGEX-2TN'Vif, pGEX-2TC'Vif, pGEX-2TΔVif, pGEX-2TTat, or pGEX-2T (Fig. 2), and co-transformants were selected in media containing chloramphenicol, ampicillin, and kanamycin.

HIV-1 protein analysis. Bacteria were propagated overnight in ampicillin and kanamycin, induced in the presence of O.1 mM IPTG for 1 h, washed, and cell pellets were lysed directly in Laemmli sample buffer containing 4% SDS. Samples were subjected to electrophoresis in a 12% SDS-polyacrylamide gel, proteins were blotted unto a Nytran membrane (BioRad, Hercules, Calif.), and blots were exposed to an AIDS patient serum or specific antibodies to visualize HIV-1 specific proteins; bound antibody was detected by luminescence (NEN DuPont, Boston, Mass). The sources and specificities of the antibodies used are: anti-CA, mouse monoclonal AG3.0 antibody which detects both mature HIV-1 CA protein and its CA precursor proteins (Simm et al., J. Virol. 69: 4582-4586, 1995) provided by J.S. Allan; anti-MA, rabbit polygonal antibody prepared against recombinant MA produced in bacteria, provided by D. Trono; anti-Vif, rabbit polygonal antibody prepared against recombinant Vif produced in bacteria, donated by D. Gabuzda. The anti-Vif antibody detects GST-Vif and GST-C'Vif (Fig. 4, anti-Vif panel).

Other cells and reagents. Lysis resistant CR10 cells were infected with HIV-1/N1T and chronic infection was established as previously described (Casareale et al., Virology 155:40-49, 1987). These cells were used as control to demonstrate the pattern of Gag and Gag-Pol proteins in infected eucaryotic cells (to compare with that seen in bacteria). The HIV-1 protease inhibitor, Ro 31-8959 was obtained from Roche Products, Ltd (London, UK) and was titered for inhibition of proteolysis by Western blotting of supernatants of infected cells.

Results

Proteolysis of Gag-Pol in bacteria in the presence of Vif. Previous studies have shown that HIV-1 protease is enzymatically active in a polyprotein precursor form in bacteria resulting in autoprotolysis of the precursor at specific protease cleavage sites (Kotler et al., J. Virol. 66:6781-6783, 1992). To test the modulation of protease activity in bacteria, Vif and control proteins were constructed as fusions with GST using a pGEX vector modified to permit co-selection of two different plasmids in the same cell (Fig. 2). The expression and molecular sizes of GST-fusion proteins were confirmed by purification of fusion proteins from bacterial extracts on glutathione-Sepharose, electrophoresis in SDS-polyacrylamide, and protein staining (Fig. 2).

Based on the known protease cleavage sites (Debouck et al., Drug Development Research 21:1-17, 1990), the predicted proteolytic products of Gag-PR and their detection by specific antibodies are shown schematically in Fig. 3A. In the first series of experiments, bacteria were induced to co-express Gag-PR and either GST-Vif (V), GST-N-terminal or C-terminal halves of Vif protein (N'V and C'V, respectively), or GST alone (G), and the Gag-PR autoprocessing products were analyzed by Western blotting using an AIDS patient serum (Fig. 3B). Co-expression with GST yielded the intact Gag-PR polypeptide of 68 kDa and the major predicted proteolytic cleavage products (Fig. 3B, lane G). HIV-1 infected human T cells contained proteins of similar mobility except that the major high molecular weight protein was p55, likely the Gag precursor $Pr55^{Gag}$ (T cells+HIV-1 lane). Co-expression of Gag-PR with either GST-Vif or GST-N'Vif, but not with GST-C'Vif, significantly altered the pattern of cleavage products detected in this system. Most strikingly, the p24 bands were diminished or absent (V and N'V lanes), although the higher molecular weight cleavage products were present at levels similar to those In tw GST control. The full range of predicted Gag-PR cleavage products was observed in the presence of GST-C'Vif (Fig. 3B, lane C'V). The GST-fusions were expressed in bacteria at similar levels, but GST alone was

roughly 10 fold more abundant (not shown), and had no effect on proteolysis (Fig. 3B). These results indicate that HIV-1 Vif selectively inhibits proteolysis of Gag-PR polyprotein in bacteria, affecting some products but not others, and that the N-terminal half of Vif is sufficient for this effect.

To confirm that the observed inhibition was Vif-specific and to identify the cleavage products affected by this activity, Gag-PR was co-expressed with each of the GST-fusions shown in Fig. 2 and analyzed the Gag-PR cleavage products by Western blotting with antibodies specific to CA, MA, Vif, and PR respectively (Fig. 4). Consistent with the results shown in Fig. 3B, GST-Vif (V lane) or GST-N'Vif (N'V) selectively inhibited Gag-PR polyprotein processing as determined by analysis of cleavage products with anti-CA and anti-MA. The release of both the HIV-1 MA and p24/p26 CA proteins from the polyprotein was reduced by co-expression of either one of the inhibitory GST-Vif polypeptides. p24/26 and p41 are major products of synthesis and autoprotolysis of Gag-PR in systems containing no inhibitory proteins (G,T,C'V, and ΔV lanes) indicating that, as previously reported (Tritch et al., J. Virol. 65:922-930, 1991), cleavage between MA and CA is the first to occur. Vif-related changes in the levels of intermediate Gag cleavage products detectable with anti-CA (anti-CA panel) or with anti-MA antibody (not shown) were not pronounced (Fig. 4) and are more easily detected during expression of different PR containing polypeptides, as shown below. Anti-Vif antiserum stained 49 kDa GST-Vif and 37 kDa GST-C'Vif proteins (V and C'V lanes). GST-N'Vif and GST-ΔVif are not detectable with this antiserum and their expression, as well as that of GST and GST-Tat proteins, was confirmed by SDS-PAGE analysis of cell extracts (Fig. 2).

Polyproteins containing PR and representing cleavage products mainly in the C' terminal portion of the precursor were visualized by anti-PR antibody staining (Fig. 4, anti-PR panel). The proteolytic patterns in the presence and absence of Vif polypeptides were similar, suggesting that all cleavages on the precursor polyprotein are not affected equally by Vif. Anti-PR staining is less

suitable than is anti-CA staining to demonstrate the cleavages flanking CA, since the N'terminal fragments would not be detected, predicted changes in the levels of PR-containing p24/26 were difficult to resolve, and a major background band obscured PR-containing p5O (Fig. 4, anti-PR panel). However the intensity and mobility of p17, a polyprotein composed of NC-p6^{Pol}-PR, are decreased in the presence of inhibitory Vif proteins, possibly reflecting secondary cleavage sites in the region following the frameshift. Co-expression of GST-C'Vif, GST-ΔVif polypeptide (ΔVif contains a naturally occurring Vif gene with a 35 base pair deletion, which generates a shift in the reading frame after amino acid 38, yielding 12 heterogenous residues before reaching a stop codon (Sakai et al., J. Virol. 67:1663-1666, 1993), HIV-1 Tat (as GST fusion), or GST protein alone did not affect autoprotolysis of Gag-PR (C'V, ΔV, T, and G lanes, respectively).

The results shown in Figs. 3 and 4 confirm that the observed inhibitory activity is specific to Vif and show that the region of Vif between amino acids 39 and 96 is required for this activity. In this model of HIV-1 Gag-Pol autoprotolysis, Vif modifies PR-mediated cleavage of HIV-1 Gag-PR primarily at the junction p2/p7NC, resulting in selective inhibition of release of mature CA proteins. The analyses shown in Fig. 4 do not exclude that other cleavage sites can also affected by Vif, but suggest that there is a ranking of Vif activity at specific sites.

PR and Vif specificity of proteolysis of Gag-Pol in bacteria. To confirm that the proteolysis of Gag-Pol and its modulation by Vif observed in bacteria are the results of effects upon the HIV-1 PR, GST-Vif and a two pairs of truncated Gag-Pol constructs were co-expressed: the first pair encoding Gag-PR in wildtype configuration or containing a mutation in the cleavage site between p6^{Pol} and PR and the second pair encoding CA-PR with or without the cleavage site mutation preceding PR (Fig. 1). The polyprotein p6^{Pol}-PR is more proteolytically active than is mature PR (Almog et al., J.Virol. 70:7228-7232, 1996). As shown in Fig. 5, proteolysis was more efficient in cells expressing either cleavage site mutant than in cells expressing wildtype Gag-PR or CA-PR, confirming that these cleavages

were carried out by HIV-1 PR; the p24/26 bands were more pronounced in lane 2 (CA_xPR) than in lane 4 (CA-PR). Expression of any of the four truncated Gag-PR vectors in the presence of GST-Vif resulted in an inhibition of cleavage, indicating that Vif acts specifically on PR-mediated proteolysis. The reduction in p24/26 in the presence of Vif (lane 7 versus lane 8) indicates that the cleavages flanking p2 are inhibited by Vif. Another Vif sensitive site is apparent in the increased intensity in the p33 band consisting of CA-p2-NC in cells expressing wildtype CA-PR in the absence of GST-Vif (lane 4) compared to the presence of GST-Vif (lane 3). That Vif affected the processing of p33 from the p50 precursor indicates that Vif can retard cleavage at the site between NC and $p6^{Pol}$. The effects of Vif were most pronounced when co-expressed with vectors carrying the less active, wildtype PR than with those carrying cleavage site mutations. The final product p24 was most easily detected in cells carrying CA_xPR or Gag_xPR in the absence of Vif (lanes 2 and 6) and was least abundant in cells expressing wildtype Gag constructs in the presence of GST-Vif (lanes 3 and 7). The presence of Vif tended to favor accumulation of incompletely processed proteins, while processing went to completion in the absence of Vif. These findings demonstrate that Vif retards proteolytic processing by PR at a number of its native cleavage sites.

Autoprotolysis of a minimum substrate in the presence of Vif. The effects of Vif on protease-mediated proteolysis at different sites are difficult to rank when several sites are present simultaneously. To determine the impact of Vif on proteolysis when only one cleavage site is available, HIV-1 protein processing in bacteria expressing $p6^{Pol}$-PR and different Vif polypeptides was examined (Fig. 6 and Fig. 7). The site separating $p6^{Pol}$ and PR is not cleaved in the context of the Gag-PR polyprotein (Fig. 4) but it is efficiently utilized as the single site present on $p6^{Pol}$-PR (Fig. 6, lane G). Mature p11 PR was undetectable in the presence of either full length GST-Vif and N'Vif indicating that they totally inhibited proteolysis at the $p6^{Pol}$-PR junction. When expressed with $p6^{Pol}$-PR, GST-Tat, GST-ΔVif, GST-C'-Vif and GST alone yielded similar ratios of precursor $p6^{Pol}$-PR

to PR, indicating that none of these polypeptides affect proteolysis more than the control, GST. These findings indicate that Vif can fully inhibit PR processing at a well-defined site.

To investigate the kinetics of processing $p6^{pol}$-PR as a function of co-expression of Vif, the experiment shown in Fig. 6 was repeated to extend the time and frequency of sample collection after induction. Examination of the time-course of product accumulation provides a more detailed view of the effects of a potential inhibitor. Because the amount of precursor polypeptide synthesized in the presence of GST-Vif or N'Vif appeared to be less than in other systems (Fig. 6), the bacterial cell extracts collected at a single timepoint for the amount of $p6^{pol}$-PR were standardized prior to electrophoresis. This facilitated the comparison of precursor to product ratios among systems. In this experiment, proteolysis of $p6^{pol}$-PR was evident within i hour of induction in the presence of GST or C'Vif and the level of product PR increased with time after induction (Fig. 7A). In contrast, despite increased levels of $p6^{pol}$-PR over time, no mature PR was observed in the presence of GST-Vif up to 2.5 h after induction. The levels of PR-containing proteins were expressed as a ratio of 11 kDa PR to total stained protein present in each lane to display the proteolysis of precursor to final product over time (Fig. 7B). The fraction of PR increased with time in the presence of GST or GST-C'Vif to approximately 40% hydrolysis, but was insignificant in the presence of GST-Vif. Consistent with previous experiments (Fig. 4-6), the processing of mature PR was also inhibited by N'Vif. These results indicate that Vif and its N'terminal half reduce the rate of protease-mediated proteolysis on this simple substrate. Mapping of the Vif regions required for inhibition of $p6^{pol}$-PR proteolysis is shown in Figure 7C.

Example 2: In Vitro Assay for Vif Modulation of Protease Activity

Methods

Purification of PR. HIV-1 PR was purified as described previously (Kotler et al., J.Virol. 66:6781-6783, 1992). Bacterial cells (E. coli BL-21 strain) expressing PR were collected by centrifugation and the pellets were suspended in 50 mM dextrose, 10 mM EDTA, 25 mM Tris (pH 8.0), and 4 mg/ml lysozyme. The suspensions were incubated for 10 min on ice and passed through a French press (2000 psi) four times. The inclusion bodies were collected by centrifugation (25,000 g for 15 min) and dissolved in 6 M guanidine-hydrochloride (pH 8.0), and acidified to pH 2.0 by the addition of TFA. The dissolved proteins were fractionated by reverse-phase high-pressure liquid chromatography on a 19X150 mm μ-Bondapack C_{18} column (Water Associates, Inc.) using a linear gradient (0-60%) of acetonitrile in the presence of 0.05% TFA. Fractions collected from the HPLC column were lyophilized and dissolved in 2M guanidine-hydrochloride, 100 mM Tris-HCl (pH 8.0), to a concentration of 200-300 μg/ml and refolded as follows: 1 vol of protein solution was diluted with 2 vol of refolding buffer (20 mM Pipes, 100 mM NaCl, 1mM EDTA, 10% glycerol), and dialyzed against refolding buffer for 4 hr at 4°C. Dialyzed fractions having the highest specific activity were collected and the protein concentration was determined, using a commercial Bio-Rad Kit.

In vitro assay substrate. The pronounced effect of Vif on Gag-PR autoprotolysis of different constructs in the studies reported above indicate that Vif can inhibit processing at many sites on Gag-Pol. This finding is consistent with an interaction of Vif with PR itself. Direct tests of such interaction are difficult to perform in cellular systems, therefore, a system for study of Vif and PR as purified proteins was established. This minimal PR enzymatic assay system consisted of recombinant PR and the chromophoric octapeptide substrate Ac-Arg-Lys-Ile-Leu[1]-Phe(NO_2)-Leu-Asp-Gly-NH_2, which mimics the cleavage site between reverse transcriptase and integrase in the Gag-Pol polyprotein (Tomaszak et al., Biochem.

Biophys. Res. Comm. 168:274-280, 1990) and is absent from the truncated Gag-PR substrates employed above (Fig. 1). HIV-1 PR cleaves the peptide between the Leu and Phe(NO_2) residues and the reaction can be measured spectrophotometrically by following the decrease in absorbance at 310 nm (Tomaszek et al., Biochem. Biophys. Res. Comm. 168:274-280, 1990).

GST-fusion protein purification. E. coli were transformed separately with each listed plasmid: pGEX-2T Vif, pGEX-2TN'Vif, pGEX-2TC'Vif, pGEX-2TΔVif and pGEX-2TTat. One hour after induction, bacteria were harvested, resuspended in a buffer containing 10 mM TrisHCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 5 mM DTT, 100μg/ml lysozyme, 1 mM PMSF on ice for 15 min, frozen and thawed, and lysed by sonication. After removal of debris, supernatant was mixed with 100 μl of 50% slurry of glutathione-Sepharose beads and incubated for 30 min at 4°C. Beads then were washed in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1 mM EDTA buffer and fusion proteins were released by incubation in 0.5 ml of 50 mM Tris-HCl, pH 8.4, 20 mM reduced glutathione, 120 mM NaCl, and 0.1% Triton X-100 (release buffer) for 4 h at room temperature. The released proteins were quantified by reference to marker proteins in SDS-PAGE and stored at -20°C in release buffer.

Spectrophotometric assay of synthetic peptide cleavage. The conditions of the assay were essentially those described (Tomaszek et al., Biochem. Biophys. Res. Comm. 168:274-280, 1990). The chromogenic synthetic peptide substrate Ac-Arg-Lys-Ile-Leu'-Phe(NO_2)-Leu-Asp-Gly-NH_2 was suspended at 250 μM in a buffer containing 80 mM sodium acetate, 1mM EDTA, 1mM dithiothreitol, and 0.8 M NaCl (pH 5.0) in a total reaction volume of 200μl. After equilibration of substrate at 37°C for 10 min, 10 ng protease alone or mixed with proteins at the indicated doses or Ro 31-8959 (Roberts et al., Science 248:358-361, 1990) were added. A_{310} was read in Beckman DU-62 spectrophotometer, the zero time point was taken at the addition of protease. The substrate, the HXB-2 protease, and purified full-length Vif and Tat proteins were all provided by the AIDS Research

and Reference Reagent Program. The GST-N'Vif and GST-C'Vif proteins were purified from bacteria transformed with either pGEX-2TN'Vif or pGEX-2TC'Vif (Fig. 2).

Results

The kinetics of substrate cleavage by PR in the presence and absence of Vif are depicted in Fig. 8. The A_{310} of substrate alone (250 μM) remained unchanged during a 35 min incubation at 37°C. The cleavage plateaued within 15 min at 37°C at 13% utilization of the substrate and it was inhibited by HIV-1 protease inhibitor Ro 31-8959 (Roberts et al., Science 248:358-361, 1990) at 10^{-5} M, confirming the PR-specificity of the reaction (Fig. 8A). The molar ratio of protease inhibitor to PR in this system was about 2000:1; no inhibition was seen at a ratio of 400:1. Addition of purified full-length Vif or GST-N'Vif also inhibited cleavage whereas Tat and GST-C'Vif proteins had no effect (Fig. 8B). Analysis of GST-N'Vif dose response in this reaction revealed maximum inhibition at a molar ratio of N'Vif to PR of 0.7:1 (Fig. 8C; GST-C'Vif had no effect at any dose tested (Fig. 8D). It is concluded that Vif (either as a purified 23 kDa protein or a purified 35 kDa GST-N'Vif protein) is a specific inhibitor of HIV-1 PR in cell free proteolytic assay using a selected synthetic peptide substrate. Since Vif efficiently blocked PR activity at approximately 1:1 molar ratio to the enzyme (Fig. 8C), and the substrate was present at a 50,000-fold excess, it is likely that Vif acts in this assay by interacting with PR. Preliminary experiments replacing HIV-1 protease with pepsin in this synthetic peptide hydrolysis assay indicated that over the concentration range employed, Vif has no effect on pepsin cleavage using 1 unit of enzyme per 50 nmoles substrate.

Example 3: Effect of Vif in Nonpermissive HIV-1 Infected Cells

Methods

The H4/CD4 cell line is a human glioma cell line which has been engineered to express CD4, making it susceptible to infection with HIV-1. An experiment to determine whether H4/CD4 can support a productive HIV-1 infection in the absence of the Vif protein was conducted. 5 x 10^4 H4/CD4 cells were transfected with 5 μg of either full-length wild-type HIV-1 proviral DNA or a mutant Vif (*vif*) proviral DNA (ΔVif-NL4-3) by $CaPO_4$ precipitation. ΔVif-NL4-3 contains a 35 bp deletion in Vif (Simm et al., J. Virol.69:4582-4586, 1992). This virus was made by exchanging the Vif open reading frame in NL4-3 with the corresponding open reading frame from N1T-E (Sakai et al., J. Virol.65:5765-5773, 1991), which is the natural clone carrying this deletion in Vif. Cells were cultured in DMEM medium with 5% FCS and antibiotics, and tested on the designated days post-infection for the expression of HIV-1 antigens by indirect immunofluorescence staining using an AIDS patient serum (Table 2).

To confirm that progeny virus made in transfected H4/CD4 cells are noninfectious, transfected cells and culture supernatants were evaluated from their infectivity by cocultivation and cell-free virus infection. 14 days after transfection, samples of transfected cells and supernatants were collected for the evaluation of virus progeny infectivity. Virus in culture supernatants used for infection was standardized by p24 content; infectious dose was 1 μg p24/10^6 cells. Cocultures were set up at a 1:10 ratio of infected to uninfected cells for vif transfectants and at a 1:50 ratio for wild-type transfectants, to account for the difference in the % HIV-1 antigen positive cells (Table 3).

Results

Transfection of wild-type viral DNA resulted in a spreading infection that encompassed 76% of cells in 17 days. In contrast, only 14% of cells expressed *vif* HIV-1 after 10 days, and the proportion of cells expressing virus remained steady

or declined thereafter, indicating the absence of viral spread in this system (Table 2).

Infected/cocultivated cells were evaluated for HIV-1 replication by testing the proportion of HIV-1 antigen expressing cells at the designated times post-infection by an indirect immunofluorescence assay using an AIDS patient serum. It was clear that the replication of Vif-mutant HIV in H4/CD4 cells results in the production of progeny virus that is noninfectious in H4/CD4 cells by both cocultivation and cell-free virus transmission assays (Table 3).

TABLE 2

Viral DNA	Expression of HIV-1 in transfected cells (% of HIV-1 antigen positive cells; days after transfection)				
	3	7	10	14	17
wt	2	28	56	64	76
vif-mutant	1	7	14	13	12

TABLE 3

Source of virus or virus-infected cells	Mode of infection	Expression of HIV-1 in infected cells (% HIV-1 antigen positive cells; days after infection)			
		3	7	10	14
wt HIV-1 DNA-transfected H4/CD4	cell-free virus infection	12	25	84	95
wt HIV-1 DNA-transfected H4/CD4	co-cultivation	54	86	88	96
HIV-1 *vif* mutant DNA-transfected H4/CD4	cell-free virus infection	<0.1	<0.1	<0.1	<0.1
HIV-1 *vif* mutant DNA-transfected H4/CD4	co-cultivation	<1	<1	<1	<1

Example 4: Blocking of HIV-1 Infection in H4/CD4 Cells by Expression of Non-functional Vif in Trans

Previous experiments demonstrated that the two cysteine residues (Cys114 and Cys133) in the Vif protein were required for Vif function (Ma et al., J. Virol. 68:1714-1720, 1994).

An experiment was performed to determine if the expression of proviral DNA encoding mutated (nonfunctional) Vif protein would interfere with the replication of wild-type virus. Mutated DNAs constructed for the experiment included a NL4-3 provirus in which both cysteines in Vif were replaced with leucines (Cys*vif*) resulting in the synthesis of full-length nonfunctional Vif that does not support production of infectious virions, and a provirus which encodes a truncated Vif protein, ΔVif, described in Example 1, infra, (Simm et al., J. Virol.

69:4582-4586, 1995). Transfections were performed using 1 μg/DNA per 10^6 cells. As shown in Table 4, cotransfection of either of the mutant proviral DNAs with wild-type DNA resulted in at least 90% reduction of the infectivity of wild-type virus, to approximately the level of expression of mutant DNAs alone, relative to a control experiment with salmon sperm DNA (ssDNA,) as indicated by the percentage of HIV-1 antigen-positive cells (Table 4), measured as described in Example 3.

Since most of the increase in the number of H4/CD4 cells expressing wild-type virus in these experiments is the result of virus spread from the initially transfected cells, these results can be interpreted to indicate that the expression of mutated Vif in trans inhibited the function of the wild-type Vif produced by wild-type virus, thereby converting the combined infection into a ΔVif-like phenotype. Owing to the significant differences between the spreading wild-type infection and the one-cycle ΔVif infection in this system (Table 2), the experiment demonstrated the utility of H4/CD4 cells for assays testing Vif-derived protease inhibitors.

TABLE 4

DNA systems	Expression of HIV-1 days after cotransfection (% of HIV-1 antigen positive cells)	
	7	10
wt + ssDNA	10	40
Cys*vif* + ssDNA	8	12
ΔVif + ssDNA	2	6
wt + Cys- *vif*	2	3
wt + ΔVif	1	4

Example 5: Synthesis of Vif-derived Protease Inhibitor Peptides

The Vif-derived protease inhibitor peptides were manually assembled on a Boc-Val-PAM resin according to the HBTU activation *in situ* neutralization protocol for BOC solid phase peptide synthesis (Barany and Merrifield in The Peptides:Analysis, Synthesis, Biology, Gross et al., eds., Vol. 2, pp. 1-225, Academic Press, New York, 1980). Rapid single coupling cycles (5 min) were used throughout the synthesis, and side chain protection was previously described (Tam et al., J. Am. Chem. Soc. 105:6442-6445, 1983). Upon completion of the chain assembly, the dinitrophenyl side chain protecting groups were removed from histidine residues prior to acidolytic cleavage. The polypeptide was then cleaved from the resin with simultaneous removal of the remaining side chain protecting groups by treatment with liquid HF containing 4% p-cresol for 1 h at 0°C. The desired polypeptide was then purified in a single step by preparative HPLC. The purified material was deemed to be >98% homogenous as indicated by both analytical HPLC and electrospray mass spectrometry. Peptides were designed from the amino acid sequence of HIV-1/N1T-A (Sakai et al., J. Virol. 65:5765-5773, 1991). The sequence and location of the peptides is shown in Table 5.

Alternatively, peptides were synthesized according to the SPPS method, using an Applied Biosystems Peptide Synthesizer, model 433A on Rink amide resin (loading 0.5 mmol/g) by standard Fmoc chemistry. They were cleaved from the resin with trifluoroacetic acid (TFA) containing 5% anisole as a scavenger, precipitated from cold ether, dissolved in water and lyophilized. Crude peptides were analyzed by reverse-phase HPLC [C3 column 5-60% acetonitrile : water gradient containing 0.1% TFA, 45 min] and characterized by TOF-MS and amino acid analysis.

TABLE 5

PEPTIDE	RESIDUES	SEQUENCE
Vif 1:	1-8	MENRWQVM (SEQ ID NO: 1)
Vif 2:	9-16	IVWQVDRM (SEQ ID NO: 2)
Vif 3:	17-29	RIRTWKSLVKHHM (SEQ ID NO: 3)
Vif 4:	30-65	YVSGKARGWFYRHHYESPHPRISSEVHIPLGD ARLV (SEQ ID NO: 4)
Vif 41:	30-45	YVSGKARGWFYRHHY (SEQ ID NO: 5)
Vif 42:	41-55	RHHYESPHPRISSEV (SEQ ID NO: 22)
Vif 43	51-65	ISSEVHIPLGDARLV (SEQ ID NO: 6)

Example 6: Formation of Vif-Protease Complexes

Methods

Protein A agarose beads were saturated with rabbit anti-PR antiserum, washed extensively with 0.1 M phosphate buffer, pH 8.5 (PB) and 250 μl of 50% suspension beads in PB were mixed with PR and washed with PBS. The suspended beads were divided into 4 equal parts and into each the following proteins were added: GST Vif, GST N'Vif, GST C'Vif and GST folistatin at 60 ng per ml, pH 8.5, and immune complexes allowed to form for 1.5 h at 4°C. The beads were washed three times in PBS, pelleted, mixed with 50 µl Laemmli buffer, boiled, and proteins were analysed by electrophoresis and Western blot, followed by staining with rabbit anti-GST antiserum with detection as described above.

Results

Using the expression vectors of GST fusion proteins previously employed to test the interaction of Vif with PR in bacteria (see Example 1), the binding of PR by Vif and its subconstructs was tested. PR was immobilized on anti-PR-Protein A

agarose beads and GST fusions with Vif, N'Vif, C'Vif, or follistatin were incubated with PR in solid phase. Proteins were eluted from the beads, run in Western blot, and the blot was stained with anti-GST. As shown in Fig. 9A GST-N'Vif was bound to PR. The major band running at approximately 5O kDa is the rabbit Ig heavy chain which was also adsorbed and eluted from beads. Unfortunately, this band obscures the detection of GST-Vif, which runs at an apparent molecular weight of 58 kDa. Fig. 9B is a repeat of the same experiment showing that N'Vif bound to the anti-PR immunoadsorbent in the presence, but not the absence of PR. The results lead to a conclusion that N'Vif directly binds PR, an activity which may account for its ability to block PR-mediated proteolysis.

Example 7: Vif-Derived Peptide Inhibition of Protease Activity

Methods

Synthetic peptides cleavage assay. The standard conditions of the assay were essentially as described previously (Arad et al., Virology 214:439-444; Kotler et al., J. Virol. 71:5774-5781, 1997): A standard 10µl reaction mixture contained 10 nmol of the natural MA/CA junction decapeptide (Val-Ser-Gln-Asn-Tyr-Pro-Ile- Val-Gln-Asn), 5 pmol purified PR and 0.1 M NaCl in 50 mM Na-phosphate buffer (pH 5.5). Mixtures were incubated at 37° C and the reaction was terminated by the addition of 90 µl guanidine-HCl (pH 8.0) to a final concentration of 6 M. Aliquots of 80 µl were analyzed by reverse phase HPLC (Vydac C_{18} column; 0-40% acetonitrile : water gradient containing 0.1% TFA) and percent cleavage was calculated as the ratio between the peak product area and the peak area corresponding to the substrate (P x100 / P+S). HIV-1 protease inhibitor RO31-8959 and Pepstatin A (Sigma) were used as controls for PR activity.

A proteolysis reaction was set up consisting of 0.5 µ pepsin in 50 mM Na acetate pH 3.5, 0.1 M NaCl, 1 mM decapeptide protease substrate corresponding to the HIV-1 MA/CA cleavage site in the presence or absence of 1 mM Vif derived

peptides. The mixture was incubated at 37°C for 30 minutes and was stopped by addition of guanidium chloride to a final concentration of 6 M. The reaction products were analysed by reverse phase HPLC and the extent of cleavage in the presence of Vif peptides is expressed relative to cleavage in the absence of Vif peptides. Pepstatin A was used as a control inhibitor of pepsin.

Results

Vif-derived peptides inhibit HIV-1 PR activity. To determine which active Vif domain(s) is capable of inhibiting PR, a set of partially overlapping peptides were synthesized, based on the sequence of HIV-1 BH10 Vif (Simon et al., J.Virol. 71:5259-5267, 1997) (Peptides 6, 7, 61, 62, 63, 71) or HIV-1/N1T-A (Sakai et al., J. Virol. 65:5765-5773, 1991) (all other peptides) (Fig.10A). The crude peptides (70-90% purity) were screened for their ability to inhibit purified PR in in vitro reactions (Fig. 10B). Peptides derived from two regions of Vif (30-65 and 78-98), namely Peptides 4, 41, 43, 6 and 7, inhibited proteolysis. The IC_{50} of Peptide 4 was in the range of 230-250μM, whereas the IC_{50} value of Peptides 6 and 7 was in the order of 110 and 25μM, respectively. Peptides 41 and 43, each composed of 15 amino acid residues, were less active than Peptide 4 (Fig. 10C). Overlapping Peptides 6 and 7, containing the Arg-Lys-Lys motive, proved to be efficient inhibitors. However, none of these peptides was as active as N'-Vif (Kotler et al., J.Virol. 71:5774-5781, 1997).

Vif-derived peptides inhibit pepsin activity. The inhibition of pepsin activity is shown in Figure 10D. Peptides 4, 5, 412 and 413 exhibited significant inhibition of pepsin-mediated proteolysis.

Example 8: Binding of HIV-1 Protease to Vif-derived Peptides

Methods

Spots of 2 μl of Vif derived peptides at 10 mg/ml, a decapeptide spanning the cleavage site between reverse transcriptase and integrase from Rous sarcoma

virus at 2 mg/ml, or GST-Vif at 60 µg per ml PBS were applied to nitrocellulose filters and allowed to air dry for 10 min. Filters were then blocked with 5% dry milk powder in PBS at least 4 h at room temperature. For exposure to PR constructs, blots were incubated in $p6^{Pol}$-PR, PR or mutated PR ($Asp^{25}Ile$) at 50 µg per ml PBS-milk for 4 h at room temperature. Filters were extensively washed with PBS and PR binding was detected by incubation with rabbit anti-PR antiserum and secondary antiserum as described above.

Vif-derived peptides/ PR binding assay. Each well of a 96 microwell ELISA plate (M 129B, Dynatech) was coated with 200 µl of a solution containing 20 µg/ml peptides in 100 mM Tris-HCl (pH 8.8) for 18 hr at 4°C. The wells were aspirated, incubated with low fat milk for 1 hr and washed with PBS containing 0.05% Tween 20. A volume of 200 µl containing 100 ng PR in 0.1M NaCl and 50 mM sodium phosphate buffer (pH 7.4) was then added to each well and the microplates were incubated for 2 hr at room temperature. In the competitive binding experiments, 100 ng of PR were incubated with 10 µg peptide, or with 14 nM RO 31-5989, for 18 hr at 4°C before adding to the coated wells. The plates were extensively washed and PR binding was determined using rabbit anti-PR serum and alkaline phosphatase-conjugated goat anti-rabbit IgG diluted 1:1000 (Sigma) as secondary antibody. Bound PR was quantified with an ELISA reader (Dynatech MR5000) at 405 nm. Antisera against HIV-1 PR were prepared by immunizing rabbits with purified PR expressed in bacteria. Monoclonal antiserum against HIV-1 CA was contributed by Dr. K Steimer and obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

Results

Binding of PR to Vif-derived peptides on filters. To further localize regions of Vif which bind PR, a set of partially overlapping peptides based on the sequence of HIV-1/NIT-A Vif (Sakai et al., J. Virol.65:5765-5773, 1991) was synthesized (Table 5). Peptides were permitted to bind to nitrocellulose filters, the

filters were incubated with PR or derivatives of PR and binding of PR was detected using anti-PR staining. Peptide 4 bound well to $p6^{Pol}$-PR, the form of PR which has been shown to be most enzymatically active (Almog et al., J.Virol. 70:7228-7232, 1996), and it also bound wildtype PR and less avidly the Asp^{25} to Ile^{25} replacement mutant of PR (Figs. 11A and 11B). The conditions of binding were varied and it was found that Peptide 4 avidly bound PR at neutral and low pH and less avidly bound at high pH. Under these conditions the GST-fusion of intact Vif did not bind PR, consistent with the immunoaffinity study shown in Fig. 9. Neither Vif peptides 1, 2, or 3 or a control peptide spanning the cleavage site between reverse transcriptase and integrase from Rous sarcoma virus was capable of binding HIV-1 PR. It was concluded the Vif Peptide 4 retains the activity of N'Vif to bind PR. Fragments of Peptide 4 were tested for their ability to bind PR.

Binding of PR to Vif-derived peptides on wells. Fifteen residue overlapping fragments of Peptide 4 were synthesized and tested for their binding to PR in an ELISA format. Peptides were permitted to bind to plastic microtiter wells and HIV-1 PR was added to wells either alone or in the presence of the cognate peptide to test specificity of binding. PR binding was detected with anti-PR (Fig. 12A). As shown in the upper panel Peptide 4 and its derivatives Peptide 41 and 43 were capable of binding PR in this format. The lower panel demonstrates that the binding of PR to Peptide 4 was specific and was quantitatively inhibited by preincubation of PR with Peptide 4. To lesser extents, similar bindings were obtained with Peptides 41 and 43. In contrast, PR binding to Peptide 42 was uninhibited by preincubation with Peptide 42, suggesting a nonsaturable, nonspecific interaction.

Peptides 4, 41, 42, 43, 6, and 7, but not Peptide 3, significantly bound PR. Binding of PR to all the absorbed peptides, except Peptide 42, was specifically inhibited by preincubation with the cognate peptides (Fig 12B). There was no specific interaction between Peptides 2, and 44 and PR.

The amount of PR required to saturate the absorbed peptides was 30-50 ng/well (Fig 12C). To determine the concentration of peptide required to block binding of PR to the absorbed peptide, 50 ng of the enzyme were preincubated with increasing concentrations of cognate peptide. Binding of PR to Peptides 4, 41, 43 and 6 was specific, as preincubation of the enzyme with each of the cognate peptides resulted in dose-dependent inhibition (Fig. 12D). In contrast, the binding of PR to Peptide 42 was not affected, indicating a nonspecific interaction.

The specific PR inhibitor Ro 31-5989, which blocks the active site of PR, was used as a control. Preincubation of PR with the inhibitor reduced the binding of the enzyme to Vif-derived peptides (Fig. 12B), showing the specificity of the reaction. Peptide 6 blocked the binding of PR to the cognate peptides more efficiently than did Ro 31-5989, whereas the PR inhibitor successfully competed with Peptides 41, 42 and 43. These results suggest that Peptide 4 and its derivatives 41, 42 and 43 compete with Ro 31-5989 for the same site on the PR molecule. In addition, the data suggest that Peptide 6 binds to a different site on PR, or that it binds to the same site with a higher affinity than does the commercial inhibitor.

Analysis of short Vif peptides in the PR-peptide ELISA binding assay (Fig. 12E) permitted further delineation of Vif regions involved in PR binding. Within the Vif region corresponding to peptide 6, octapeptide 62 but not 61 or 63 showed strong and specific PR binding, indicating that PR binding activity resides between aa 80 and 87. Likewise, within the region covered by peptide 41, peptides 412 and 413 but not 411 bound PR specifically, indicating that another PR binding region in Vif maps to aa 32-43. In contrast, the region corresponding to peptide 43 is largely devoid of specific PR binding activity.

Example 9: Inhibition of Protease Activity and Virion Maturation by Vif-Derived Peptides in an Eucaryotic Expression System

Methods

The results presented in Example 8 indicate that at least some of the interaction of Vif with PR is preserved in small peptides fragments of the N-terminal domain. It was reasoned that peptides of this size may be taken up by eucaryotic cells in culture, offering the means to test their activity of PR function in cells susceptible to HIV-1 infection. To maximize the resolution of PR activity, the vaccinia vector of HIV-1 Gag-Pol, vVK-1, designed by Dr. B. Moss and colleagues (Karacostas et al., Proc. Natl. Acad. Sci. USA 86:8964-8967, 1989), was used. vVK-1 infection of many human cell types, including primary T lymphocytes, results in high levels of Gag-Pol production within 24 h of infection and also permits the native PR-mediated processing of Gag-Pol and the assembly of virion-like particles which can be isolated from the cell supernatants. The effects of Vif derived peptides on Gag-Pol processing were tested in two human cell lines, Hut-78 and CEM, which are T leukemia cell lines, and phytohemagglutinin stimulated peripheral blood lymphocytes (PBL). PBL have been shown to be the cell type most dependent upon Vif for productive HIV-1 infection.

Cell lines were obtained from the ATCC. CV-1 monkey kidney cells were cultured in DMEM supplemented with 5% fetal calf serum (FCS) as monolayers. Hut-78 and CEM human T leukemia cells were cultured in suspension in RPMI 1640 supplemented with 10% FCS. Peripheral blood was obtained from healthy donors by venipuncture and lymphocytes were isolated by Ficoll-Paque density sedimentation. Peripheral blood lymphocytes (PBL) were cultured in RPMI supplemented with 10% FCS and 5 μg per ml phytohemagglutin.

Vaccinia virus expressing HIV-1 gag-pol, vVK-1, was kindly supplied by Dr. B. Moss (NIH). For propagation of the vaccinia vector, subconfluent CV-1 cultures were infected with vVK-1 at an MOI (multiplicity of infection) of 1.

Following absorption for one hour at 37°C, cells were cultured for 24-48 hours prior to harvest of virus. At the appearance of cytopathic effects, CV-1 cells and culture supernatants were collected and sonicated to prepare virus stocks. For assays of peptide activity on HIV-1 Gag-Pol structure, T cell lines or PBL cultures were infected with vVK-1 at an MOI of 5 in the presence of the designated peptide at 100 μg/ml. One hour after exposure to virus cells were washed and then were incubated in complete medium containing the same concentration of peptide for 24 h prior to harvesting the cells and medium for HIV-1 protein expression.

Cells were pelleted by sedimentation at 5000xg for 10 min. Pellets of 2 x 10^6 cells were resuspended in 0.2 ml Laemmli loading buffer, boiled, and 15-20 µl were electrophoresed per system. Virus-like particles were isolated from cell supernatants by centrifugation at 100,000xg for 45 min. Pellets containing particulate matter representing virus-like particles from 2 x 10^6 cells were resuspended in 50 µl of loading buffer, boiled, and 20 µl was electrophoresed in 12% polyacrylamide gels. Western blots of gels were prepared by standard techniques and were stained with AG3.0 monoclonal anti-CA antibody, horseradish peroxidase coupled anti-IgG, and a luminescent substrate.

Results

Infection of human T-cell lines CEM and Hut 78, and PHA-stimulated PBL with vVK-1, resulted in high levels of Gag protein production within 24 hr, allowing the natural PR-mediated processing of viral precursors and the release of virus-like particles. As shown in Fig. 13, extracts prepared from vVK-1 infected cells and from the particles exported to the medium contained completely or partially processed Gag proteins, namely, $p55^{Gag}$, MA-CA p41 and CA p24, as determined by Western blot. Extracts prepared from vVK-1-infected Hut 78 and CEM cells incubated for 24 hr with Peptides 42 and 43 contained the same HIV-1 polyproteins present in the control vVK-1 infected cells. However, lysates of cells

incubated with Peptides 41, 6 and 7 contained the unprocessed MA-CA and $p55^{Gag}$ polyproteins and only small amounts of mature CA protein (panels A and C).

Production and autoprocessing of HIV-1 precursors in vVK-1-infected PBL were not dramatically affected by the Vif-derived peptides (panel E). However, particles released from PBL (panel F) and from the transformed cell-lines Hut 78 and CEM (panels B and D, respectively) following treatment with Peptides 41, 6 and 7, contained mostly unprocessed Gag polyproteins and only minor fractions of mature CA protein. Peptide 43 exerted an effect similar to that of Peptides 41, 6, and 7 on particles released from PBL (panel F), but did not affect, or only slightly affected the particles released from Hut 78 and CEM cells. These results clearly demonstrate that only peptides derived from residues Tyr30-Val65 and Asp78-Val98 inhibit the processing of the viral precursors expressed in human cells. Differences in the penetration and stability of the linear Vif-derived peptides may have caused the various effects on viral precursor processing observed in the three cells types used.

Example 10: Vif-Derived Peptides Reduce The Production of Infectious Viruses

Methods

Five days postinfection of Hut 78 cells with HIV-1_{IIIB} at an M.O.I. of 0.5, cells were washed and incubated for 1 hr with a mixture of 1 ml of RPMI 1640 medium and 0.5 ml water containing 1 mg peptide. Cell suspensions were diluted with RPMI medium to a final concentration of 100 µg/ml peptide and incubated for 48 hr. Cells and viruses were harvested and analyzed as described in Example 9. HIV-1_{IIIB} was kindly supplied by Dr. Wainberg (Lady Davis Institute, Montreal, Canada).

Results

Treatment of cells with Vif-derived peptides did not influence the autoprocessing of Gag polyproteins, as determined by Western blots of the cell lysates (Fig. 14A). On the other hand, Peptides 41, 6 and 7, but not Peptides 42 and 43, prevented the release of particles containing CA into the culture media (Fig. 14B). Peptides 41, 6 and 7 may have caused undetectable inhibition of Gag and Gag-Pol autoprocessing in the cells, which was sufficient to interfere with assembly and/or release of particles, as shown previously for other PR inhibitors (Kaplan et al., J.Virol. 67:4050-4055, 1993; Kaplan et al., J.Virol. 67:6782-6786, 1994).

A quantity of $7.5x10^4$ chronically infected Hut 78 cells/well (5 days postinfection) were cultivated for 9 days in a 96-well-microwell-plate in 200 µl of RPMI 1640 containing 100 µg/ml of Vif-derived peptides. Half of the culture medium was replaced daily with fresh medium containing 50 µg/ml of the tested peptide. Aliquots removed at the indicated days were quantified for p24 CA antigen, using a Vironostika HIV-1 Antigen Microelisa System (Organon Teknika, USA). Figure 15A shows that prolonged treatment of HIV-1_{IIIB} chronically infected Hut 78 cells with Peptides 41 and 6, but not with Peptides 42 and 43, caused a reduction in the total amount of particulate and soluble p24 antigen present in the culture media. Similarly, treatment of newly infected Hut 78 cultures (at an HIV-1_{IIIB} M.O.I. of 0.1) with Peptides 41, 6, and 7 for 9 days reduced the release of CA antigen into the media, indicating less virus propagation in these cells (Fig. 15B).

Media harvested on day 9 from the chronically infected treated cultures were tested $CD4^+$ HeLa cells (Chesebro et al., J.Virol. 62:3779-3788, 1988). The reduced concentrations of p24 CA antigen in the culture media following treatment with Peptides 41 and 6 correlated with the decreased production of infectious virions: Peptides 41 and 6 reduced the titer of the infectious virus by three orders of magnitude, whereas Peptide 7 lowered the titer by 6- to 10-fold. Thus, the data indicates that Peptides 41 and 6, and to a lesser extent Peptides 43 and 7, interfere

with the autoprocessing of viral precursors in human cells infected with vVK-1 or HIV-1 and cause a significant reduction in the number of infectious virions released from the HIV-1 infected cells.

Example 11: Inhibition of HIV-1 Replication in PBL by Vif-Derived Peptides

Methods

PBL were stimulated by cultured with phytohemagglutin-interleukin 2 and were infected with 0.1 pg p24 per cell HIV-/ADA. After infection, cells were cultured with 100 μg Vif peptide A (residues 21-65 HIV-1/N1T-A Vif), Vif peptide B (residues 41-65 N1T-A Vif), control peptide, or 1 μM Ro 31-8959 (saquinavir). The extent of HIV-1 infection was monitored by measurement of extracellular core antigen p24by ELISA on days 3, 7 and 10 after infection.

PBL were also cultured, infected, and exposed to peptides as described above, except that graded doses of peptides were used. The Vif peptide employed was N1T-A Vif residues 41-65 (Peptide B). HIV-1 infection was monitored by measurement of extracellular p24 on days 7, 9 and 12 after infection.

Results

In two experiments the Vif peptides, but not control peptides, reduced p24 production four (see Fig. 16A) and two orders of magnitude, respectively. PBL viability and growth were not affected.

There was no inhibition of infection by the control peptide at any concentration; the Vif derived peptide blocked HIV-1 replication by five orders of magnitude at the highest dose yielding inhibition indistinguishable from the Roche HIV-1 protease inhibitor, Ro 31-8959 (Fig. 16B). At the intermediate dose the Vif peptide inhibited infection 30-fold, and the lowest dose of peptide detectably inhibited HIV-1 replication at the earliest time point. PBL growth and viability were not affected.

SEQUENCE LISTING

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(D) SOFTWARE: FastSEQ for Windows Version 2.0b

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: 20-MAY-1998
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids

(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Glu Asn Arg Trp Gln Val Met
1 5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ile Val Trp Gln Val Asp Arg Met
1 5

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Arg Ile Arg Thr Trp Lys Ser Leu Val Lys His His Met
1 5 10

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Tyr Val Ser Gly Lys Ala Arg Gly Trp Phe Tyr Arg His His Tyr Glu
1 5 10 15
Ser Pro His Pro Arg Ile Ser Ser Glu Val His Ile Pro Leu Gly Asp
20 25 30
Ala Arg Leu Val
35

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Tyr Val Ser Gly Lys Ala Arg Gly Trp Phe Tyr Arg His His Tyr
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Ile Ser Ser Glu Val His Ile Pro Leu Gly Asp Ala Arg Leu Val
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Tyr Val Ser Gly Lys Ala Arg Gly
1 5

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Gly Lys Ala Arg Gly Trp Phe Tyr
1 5

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Gly Trp Phe Tyr Arg His His Tyr
1 5

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Ile Ser Ser Glu Val His Ile Pro Leu
1 5

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Val His Ile Pro Leu Gly Asp Ala
1 5

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Pro Leu Gly Asp Ala Arg Leu Val
1 5

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Leu Gly Asp Ala Arg Leu Val Ile Thr Thr Tyr Trp Gly Leu His Thr
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Ile Thr Thr Tyr Trp Gly Leu His Thr Gly Glu Arg Asp Trp His Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Asp Trp His Leu Gly Gln Gly Val Ser Ile Glu Trp Arg Lys Lys
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Asp Trp His Leu Gly Gln Gly Val
1 5

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Leu Gly Gln Gly Val Ser Ile Glu
1 5

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Val Ser Ile Glu Trp Arg Lys Lys
1 5

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Glu Trp Arg Lys Lys Arg Tyr Ser Thr Gln Val
1 5 10

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Glu Trp Arg Lys Lys Arg Tyr Ser
1 5

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Tyr Val Ser Gly Ala Arg Gly Trp Phe Tyr Arg His His Tyr Glu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Arg His His Tyr Glu Ser Pro His Pro Arg Ile Ser Ser Glu Val
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Asp Ala Arg Val Ile Thr Thr Tyr Trp Gly Leu His Thr Gly
1 5 10

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Ile Thr Thr Tyr Trp Gly Leu His Thr Gly Glu Arg Asp Trp His
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Leu Gly Gln Gly Val Ser Ile Lys Trp Arg Lys Lys Arg Tyr Ser
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Met Glu Asn Arg Trp Gln Val Met Ile Val Trp Gln Val Asp Arg Met
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Trp His Ser Leu Ile Lys Tyr Leu Lys Tyr Lys Thr Lys Asp Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Tyr Val Ser Lys Lys Ala Arg Gly Trp Phe Tyr Arg His His Tyr Glu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Arg His His Tyr Glu Ser Thr Asn Pro Arg Ile Ser Ser Glu Val
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

Tyr Phe Asp Cys Phe Glu Asp Ser Ala Ile Arg Lys Ala Leu Leu Gly
1 5 10 15
His Ile Val Ser Pro Arg Cys
20

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Ser Leu Gln Tyr Leu Ala Leu Ala Ala Leu Ile
1 5 10

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

Met Glu Asn Arg Xaa Xaa Val Met Ile Val Trp Gln Xaa Asp Arg Met
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Tyr Xaa His His Tyr
1 5

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

Thr Tyr Trp Gly Leu
1 5

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Asp Pro Xaa Leu Ala Asp
1 5

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

Tyr Phe Asp Cys Phe
1 5

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

Leu Gln Tyr Leu Ala Leu Xaa Xaa Pro
1 5

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

Pro Pro Leu Pro Xaa Val Xaa Lys Leu Thr Glu Asp Arg Trp Asn Lys
1 5 10 15
Pro

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 96 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

```
Met Glu Asn Arg Trp Gln Val Met Ile Val Trp Gln Val Asp Arg Met
 1               5                  10                  15
Arg Ile Arg Thr Trp Lys Ser Leu Val Lys His His Met Tyr Val Ser
            20                  25                  30
Gly Lys Ala Arg Gly Trp Phe Tyr Arg His His Tyr Glu Ser Pro His
        35                  40                  45
Pro Arg Ile Ser Ser Glu Val His Ile Pro Leu Gly Asp Ala Arg Leu
    50                  55                  60
Val Ile Thr Thr Tyr Trp Gly Leu His Thr Gly Glu Arg Asp Trp His
65                  70                  75                  80
Leu Gly Gln Gly Val Ser Ile Lys Trp Arg Lys Lys Arg Tyr Ser Thr
                85                  90                  95
```

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 96 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

```
Gln Val Asp Pro Glu Leu Ala Asp Gln Leu Ile His Leu Tyr Tyr Phe
 1               5                  10                  15
Asp Cys Phe Ser Asp Ser Ala Ile Arg Lys Ala Leu Leu Gly His Ile
            20                  25                  30
Val Ser Pro Arg Cys Glu Tyr Gln Ala Gly His Asn Lys Val Gly Ser
        35                  40                  45
Leu Gln Tyr Leu Ala Leu Ala Ala Leu Ile Thr Pro Lys Lys Ile Lys
    50                  55                  60
Pro Pro Leu Pro Ser Val Thr Lys Leu Thr Glu Asp Arg Trp Asn Lys
65                  70                  75                  80
Pro Gln Lys Thr Lys Gly His Arg Arg Ser His Thr Met Asn Gly His
                85                  90                  95
```

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 50 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

```
Met Glu Asn Arg Trp Gln Val Met Ile Val Trp Gln Val Asp Arg Met
 1               5                  10                  15
Arg Ile Arg Thr Trp Lys Ser Leu Val Lys His His Met Tyr Val Ser
            20                  25                  30
Gly Lys Ala Arg Gly Trp Asn Lys Phe Arg Ser Thr His Pro Thr Arg
        35                  40                  45
Gly Cys
    50
```

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

```
Trp Lys Ser Leu Val Lys His His Met Tyr Val Ser Gly Lys Ala Arg
 1               5                  10                  15
Gly Trp Phe Tyr Arg His His Tyr Glu Ser Pro His Pro Arg Ile Ser
            20                  25                  30
Ser Glu Val His Ile Pro Leu Gly Asp Ala Arg Leu Val
        35                  40                  45
```

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

```
Arg His His Tyr Glu Ser Pro His Pro Arg Ile Ser Ser Glu Val His
 1               5                  10                  15
Ile Pro Leu Gly Asp Ala Arg Leu Val
            20                  25
```

CLAIMS

1. A protease inhibitor which inhibits lentiviral or retroviral replication, said inhibitor comprising a peptide or polypeptide having an amino acid sequence which substantially corresponds to an amino acid sequence in a lentiviral Vif protein.

2. The inhibitor of Claim 1, in which the lentiviral Vif protein is the Vif protein of HIV-1.

3. The inhibitor of Claim 1, in which the lentivirus is HIV-1.

4. The inhibitor of Claim 1, having a sequence selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 25, SEQ ID NO: 39, SEQ ID NO: 42 and SEQ ID NO: 43, or combinations thereof.

5. A composition comprising an inhibitor of Claim 1 and a carrier, containing an amount of said inhibitor effective to inhibit lentiviral or retroviral replication.

6. A composition comprising an inhibitor of Claim 4 and a carrier, containing an amount of said inhibitor effective to inhibit lentiviral or retroviral replication.

7. A method of inhibiting lentiviral or retroviral replication, comprising administering the composition of Claim 5 to cells infected with a lentivirus or other retrovirus.

8. A method of inhibiting lentiviral or retroviral replication, comprising administering the composition of Claim 6 to cells infected with a lentivirus or other retrovirus.

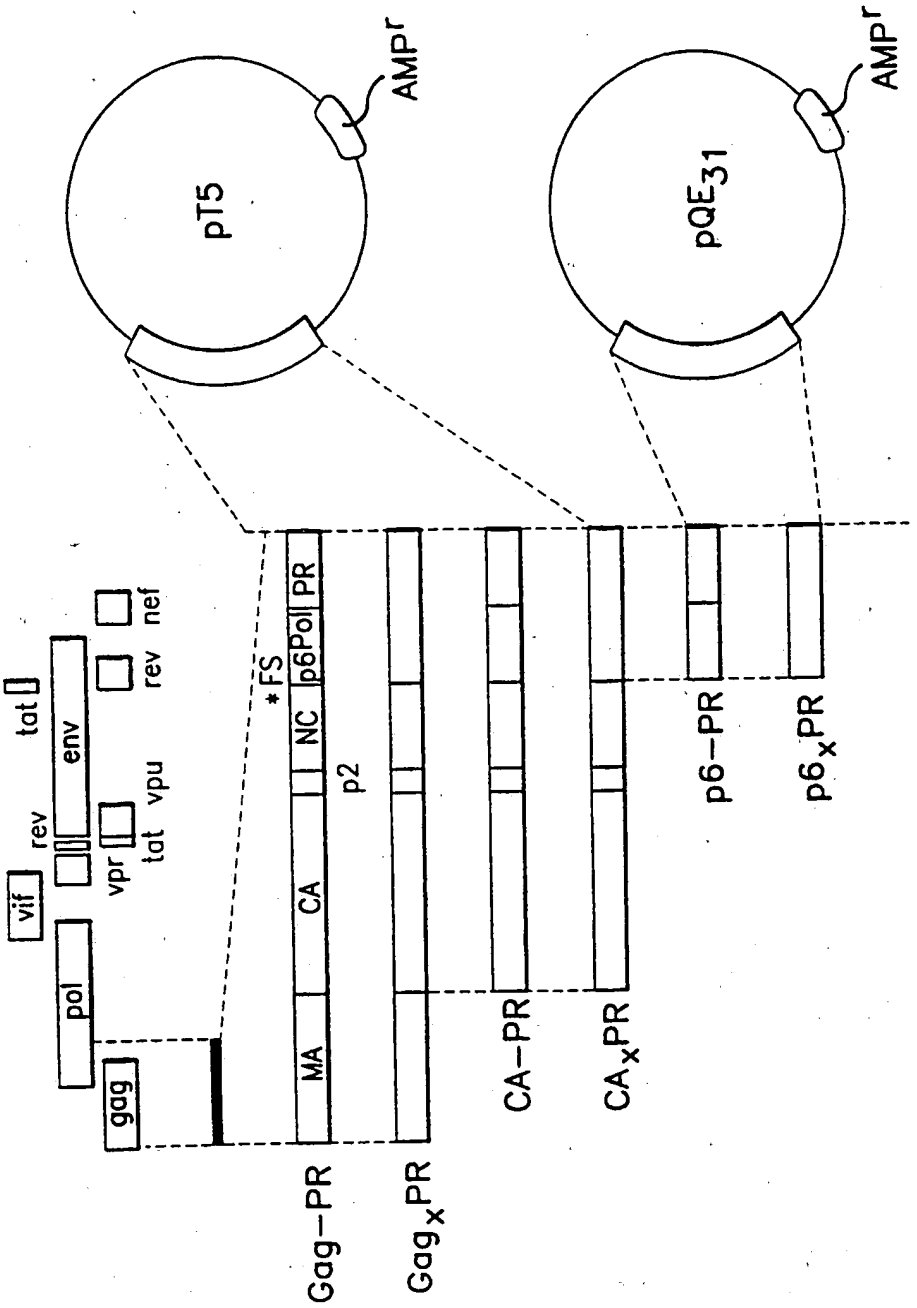
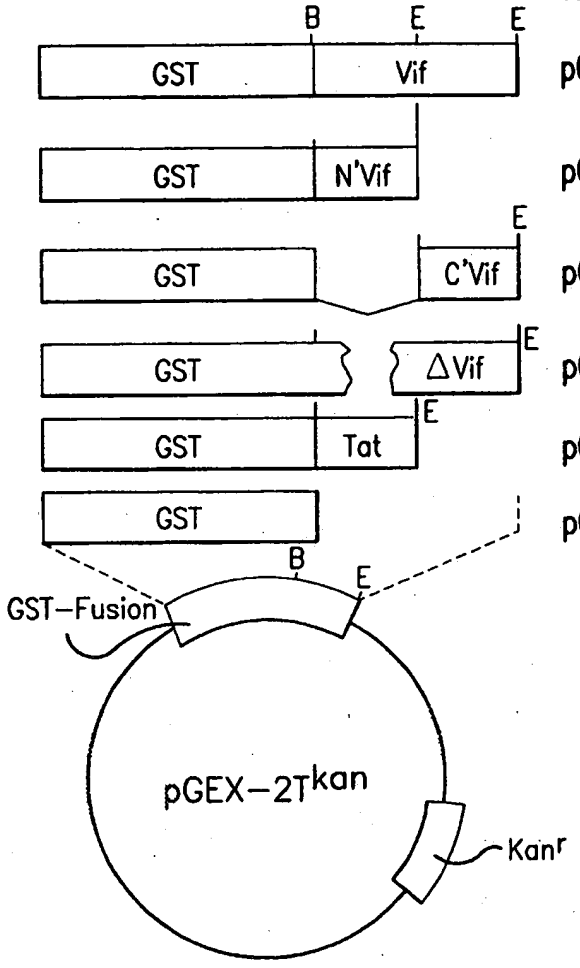

pT5
AMP^r
pQE31
AMP^r
vif
rev
env
tat
rev
nef
vpr
tat
vpu
pol
gag
*FS
MA
CA
NC
p6
Pol
PR
p2
Gag–PR
Gag_xPR
CA–PR
CA_xPR
p6–PR
p6_xPR

FIG.1



VECTOR	PROTEIN	SIZE (kDa)
pGEX–2TVif	GST–Vif	49
pGEX–2T N'Vif	GST–N'Vif	35
pGEX–2T C'Vif	GST–C'Vif	36
pGEX–2T ΔVif	GST–Δ Vif	32
pGEX–2T Tat	GST–Tat	36
pGEX–2T	GST	27

FIG.2A

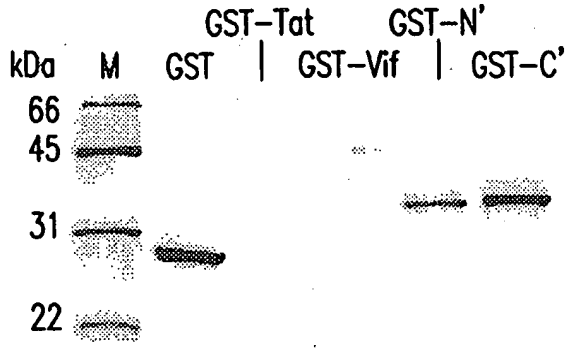


FIG.2B

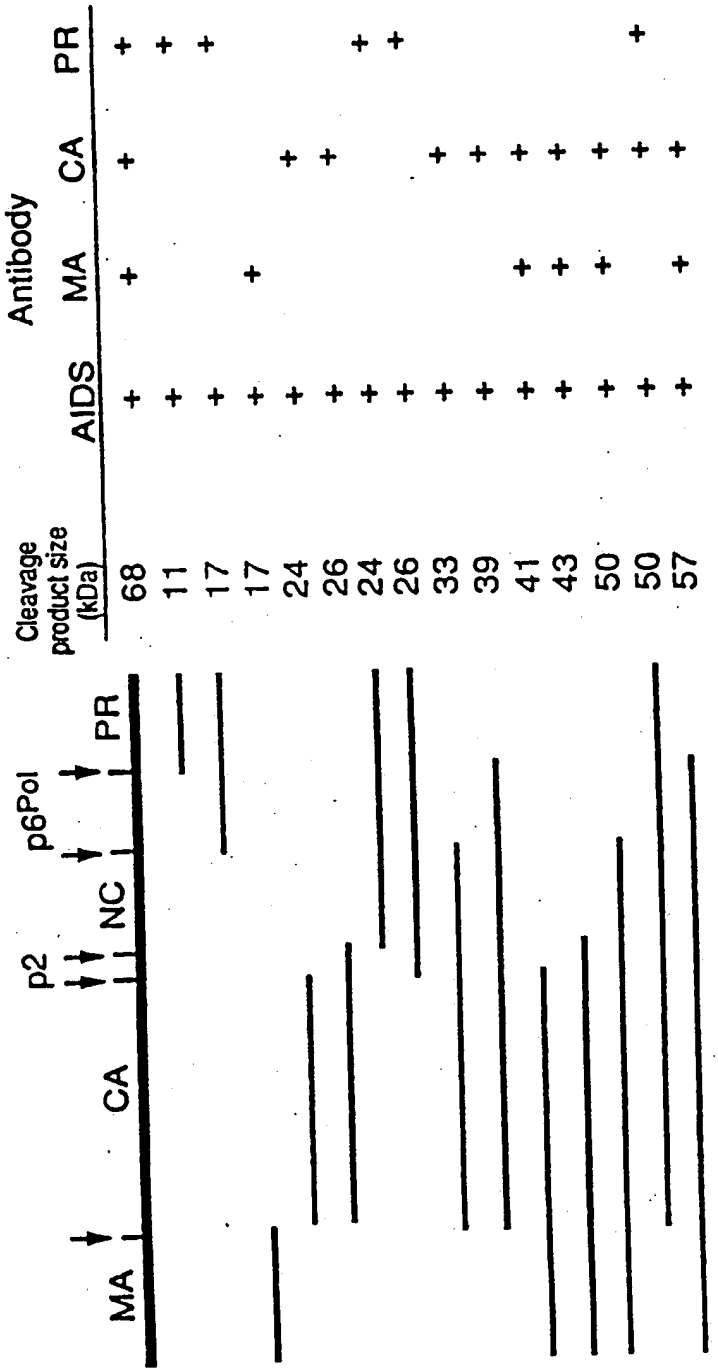
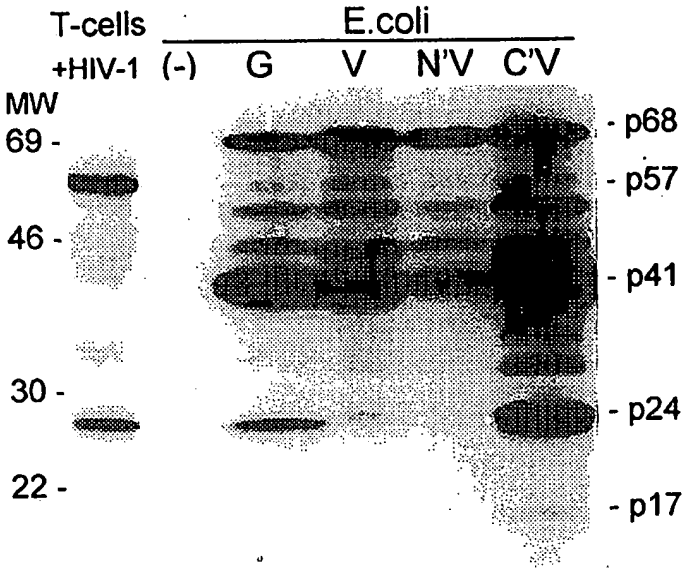

Antibody
AIDS
MA
CA
PR
Cleavage product size (kDa)
68
11
17
17
24
26
24
26
33
39
41
43
50
50
57
MA
CA
p2
NC
p6Pol
PR

FIG. 3A



AIDS serum

FIG.3B

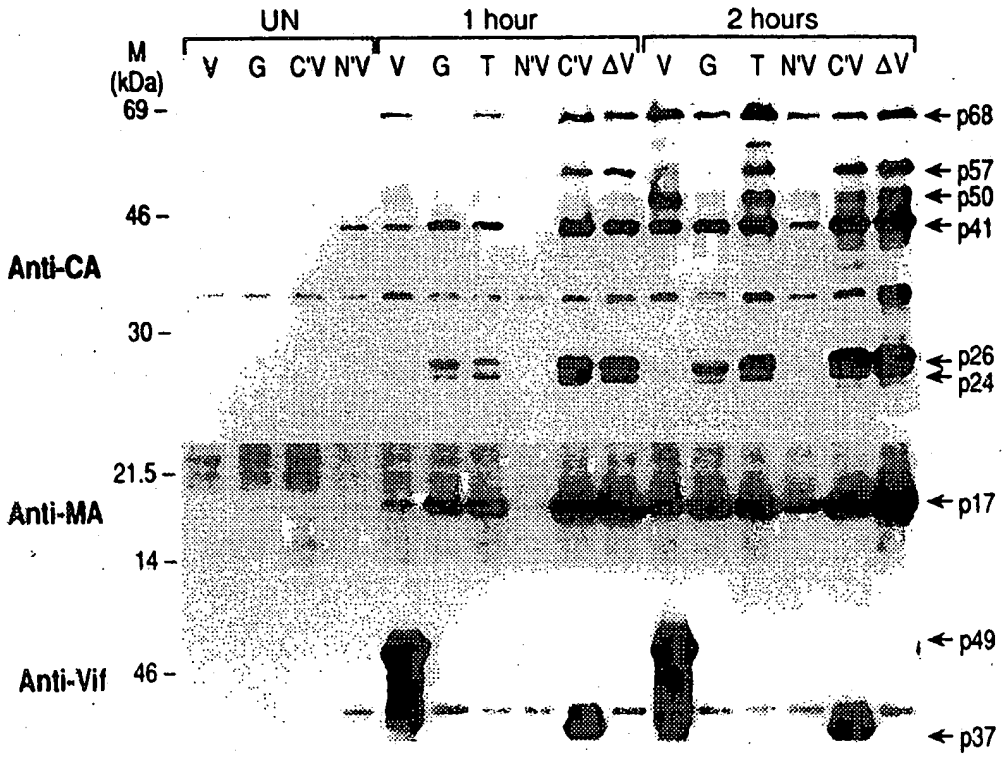
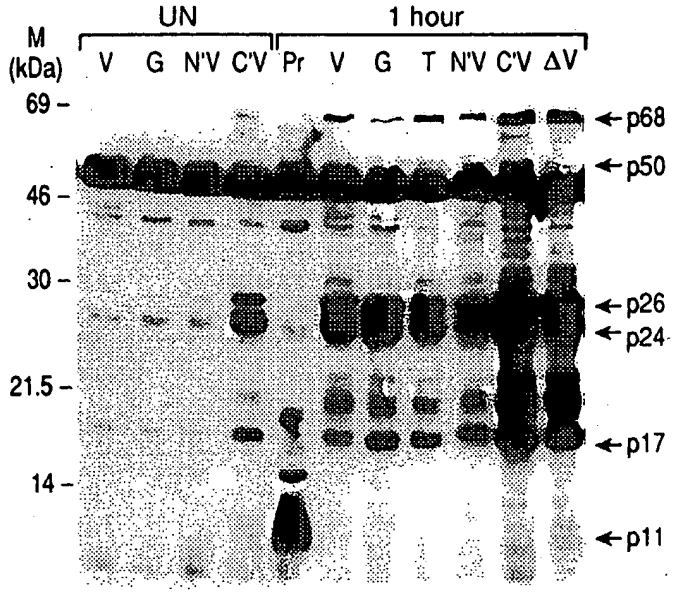
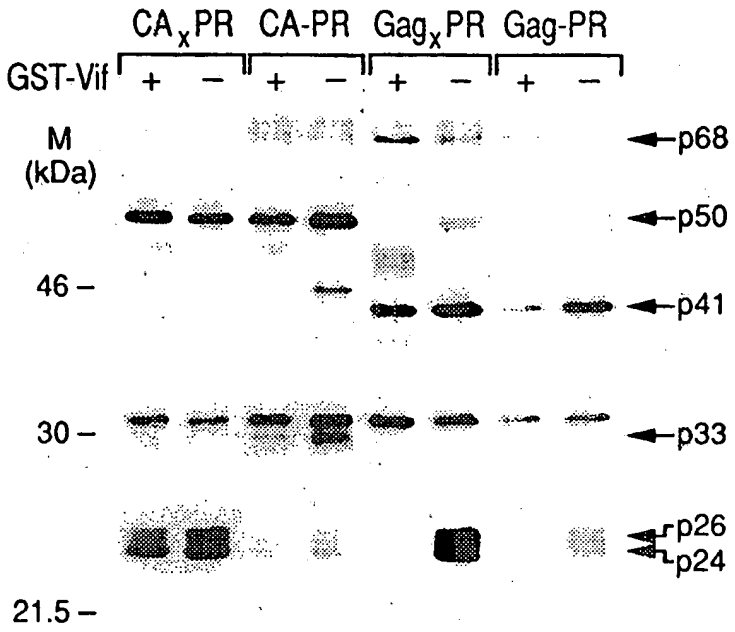

UN
1 hour
2 hours
M (kDa)
V G C'V N'V V G T N'V C'V ΔV V G T N'V C'V ΔV
69 –
46 –
30 –
21.5 –
14 –
46 –
Anti-CA
Anti-MA
Anti-Vif
p68
p57
p50
p41
p26
p24
p17
p49
p37

FIG.4A



Anti-PR

FIG.4B


CA$_x$PR
CA-PR
Gag$_x$PR
Gag-PR
GST-Vif
+ − + − + − + −
M
(kDa)
46 –
30 –
21.5 –
p68
p50
p41
p33
p26
p24

Anti-CA

FIG.5

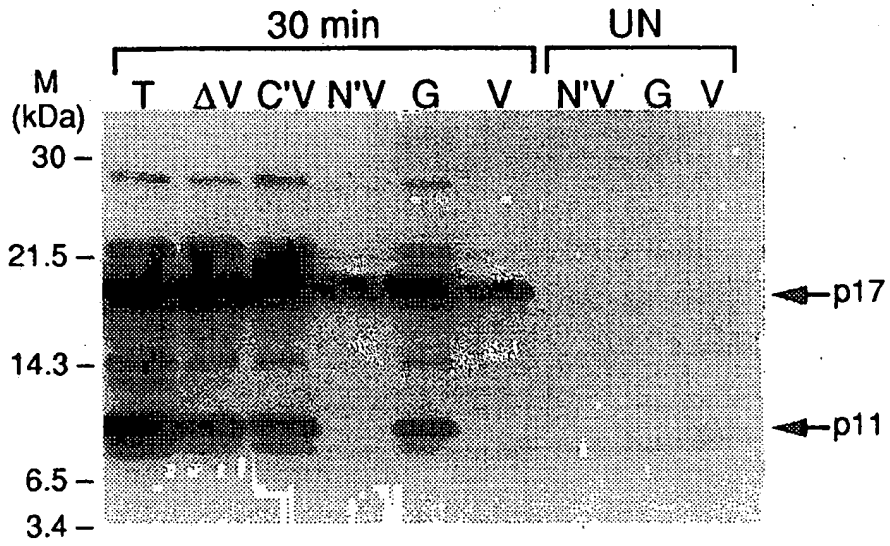


FIG.6

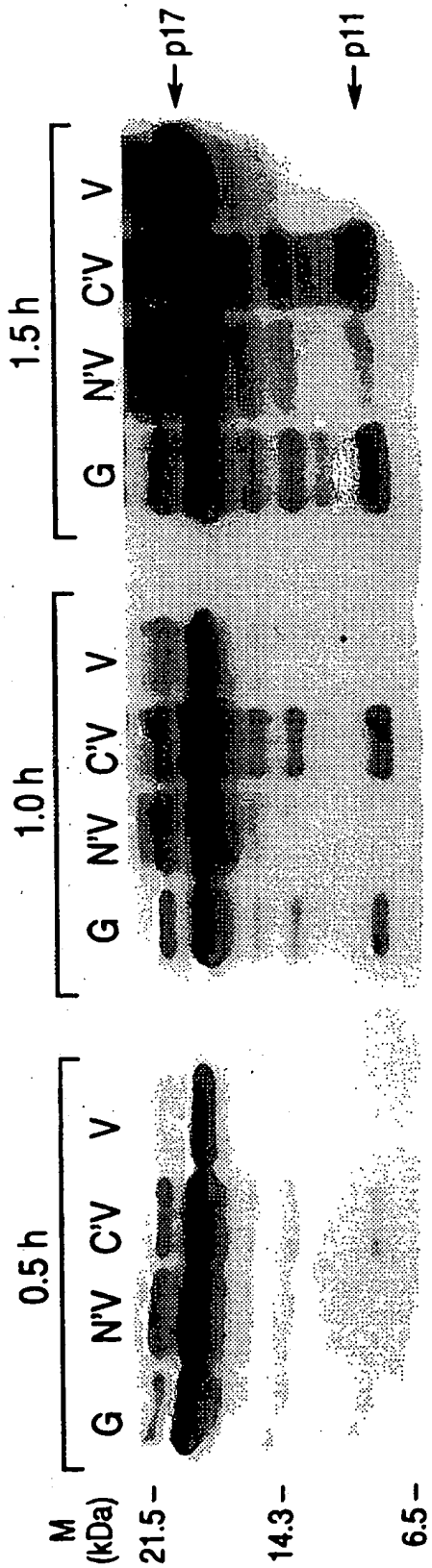


FIG. 7A

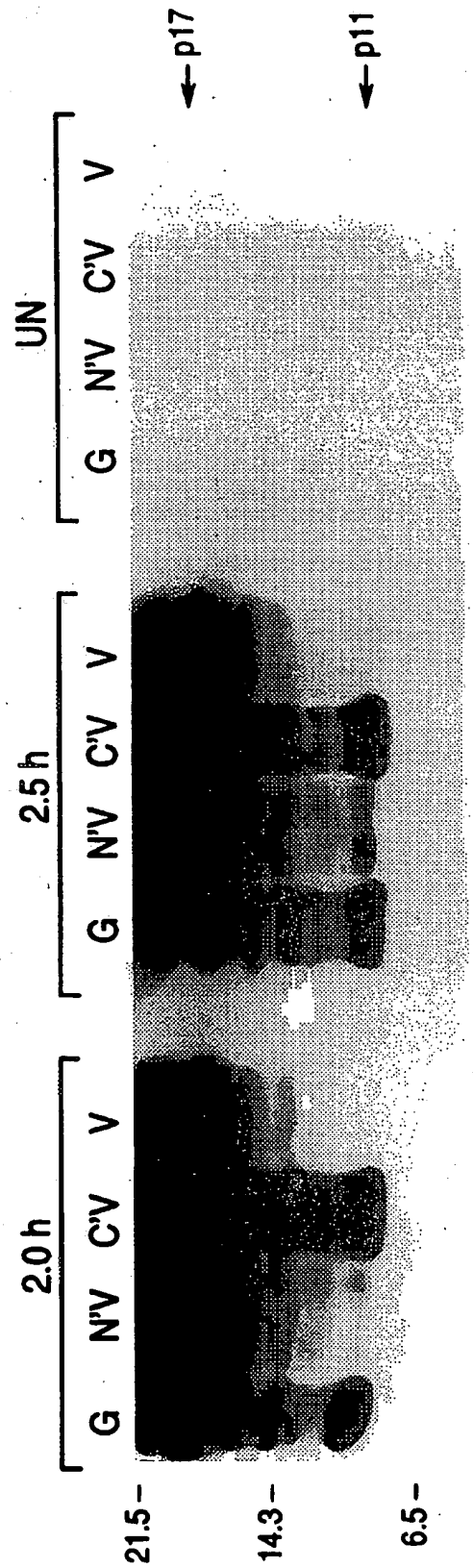


FIG.7B

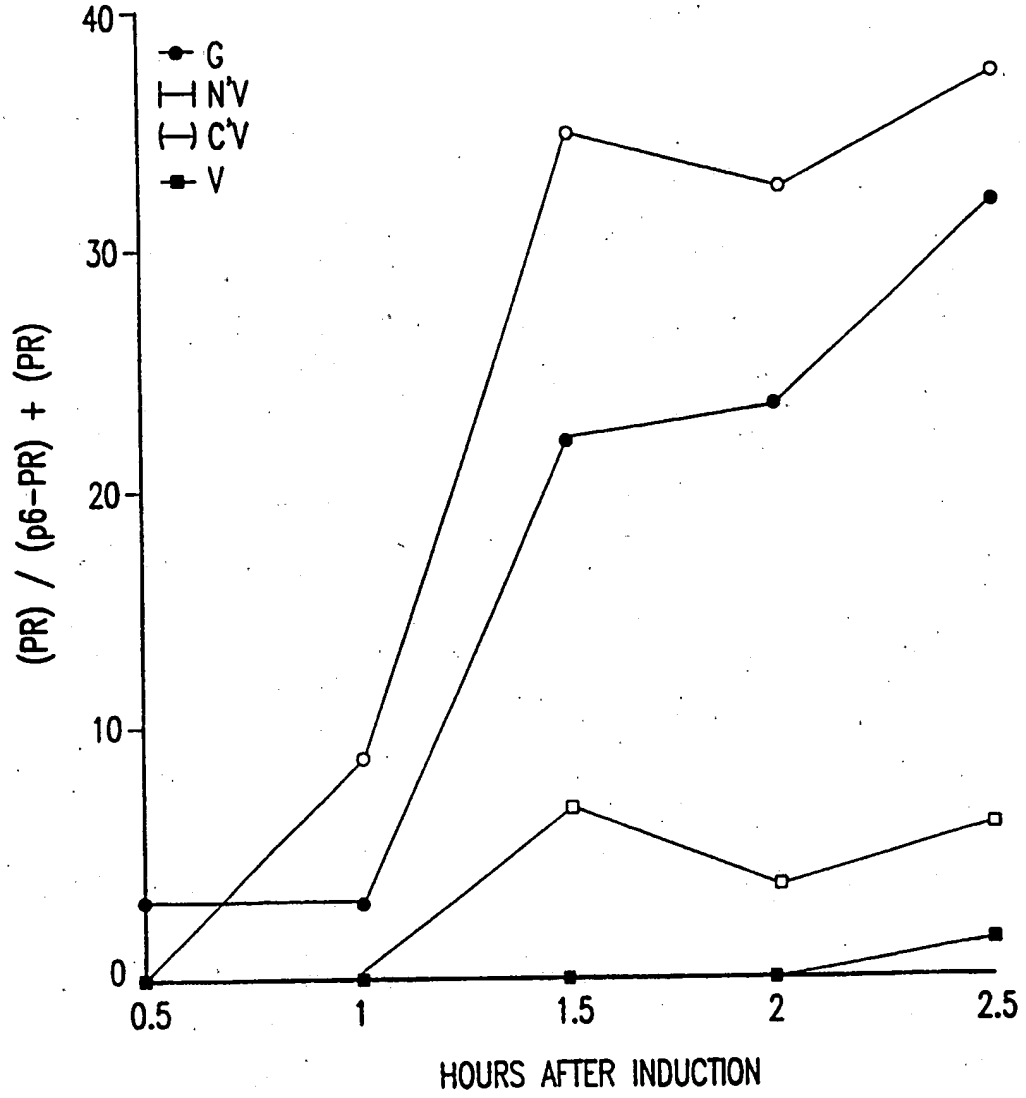

40
30
20
10
0
G
N'V
C'V
V
(PR) / (p6-PR) + (PR)
0.5
1
1.5
2
2.5
HOURS AFTER INDUCTION

FIG.7C

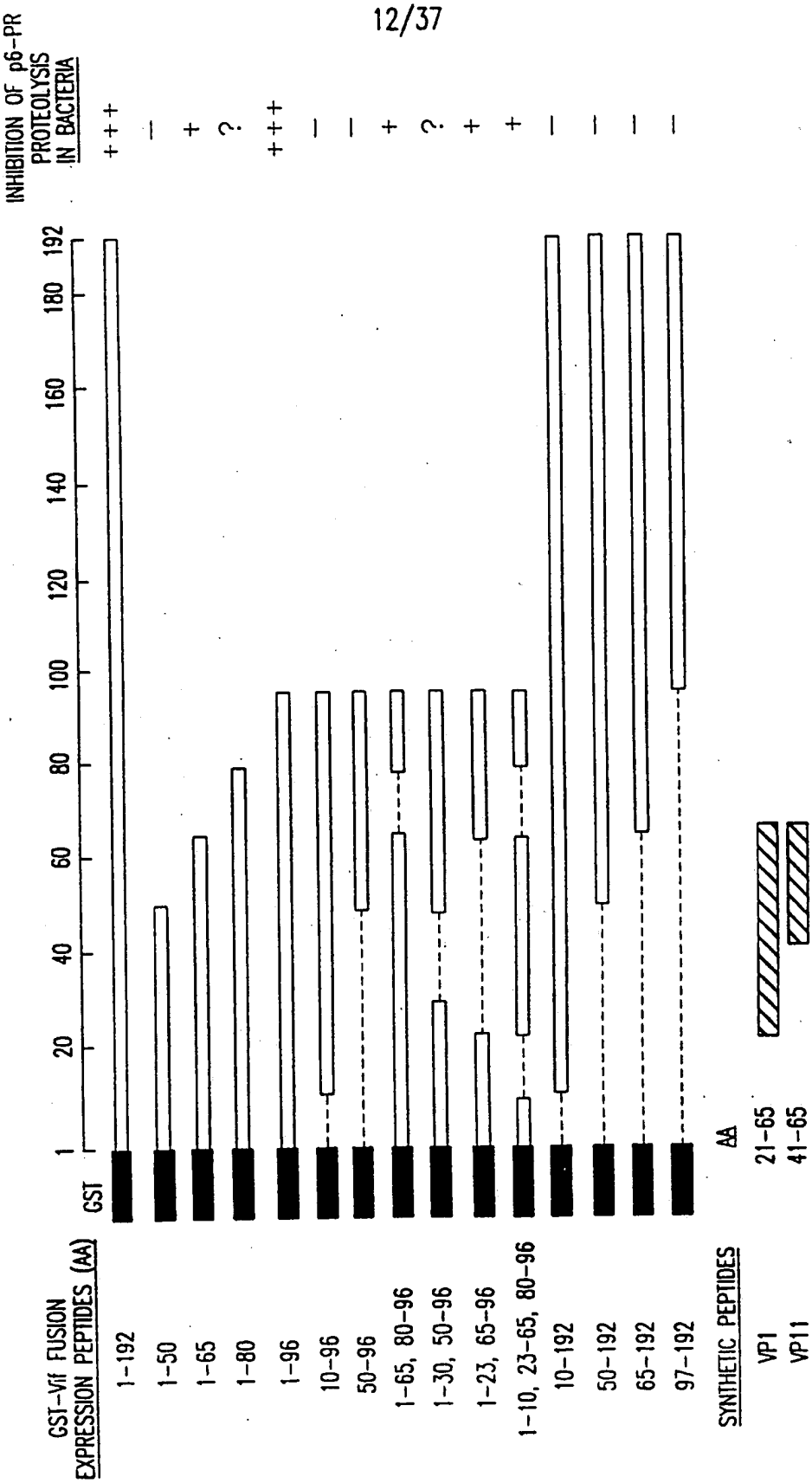

INHIBITION OF p6-PR PROTEOLYSIS IN BACTERIA
+++
–
+
?
+++
–
–
+
?
+
+
–
–
–
–
GST-Vif FUSION EXPRESSION PEPTIDES (AA)
1-192
1-50
1-65
1-80
1-96
10-96
50-96
1-65, 80-96
1-30, 50-96
1-23, 65-96
1-10, 23-65, 80-96
10-192
50-192
65-192
97-192
GST
1
20
40
60
80
100
120
140
160
180
192
AA
SYNTHETIC PEPTIDES
VP1
VP11
21-65
41-65

FIG.7D

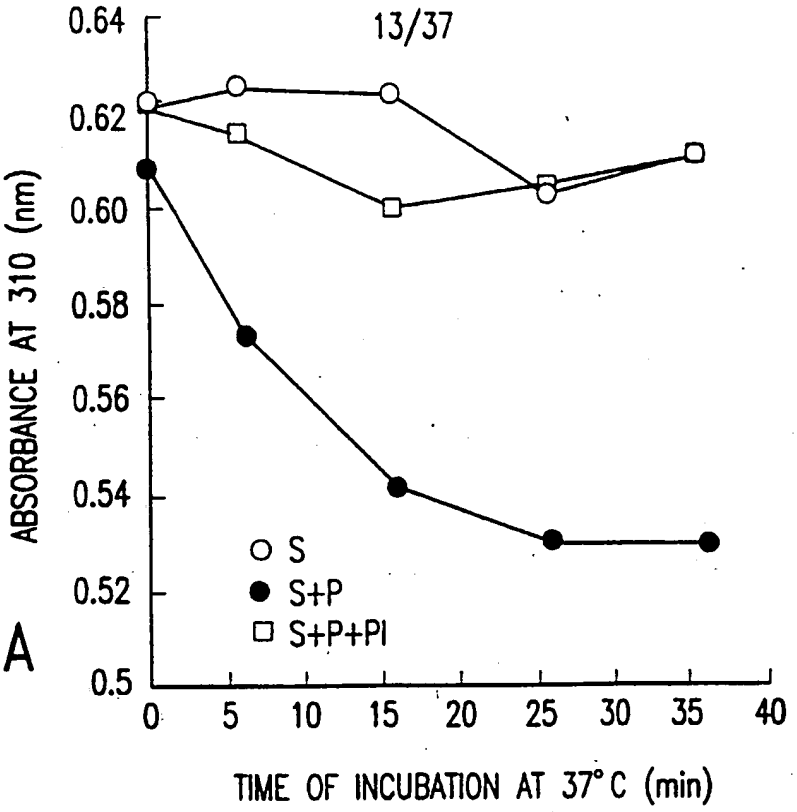

0.64
0.62
0.60
0.58
0.56
0.54
0.52
0.5
ABSORBANCE AT 310 (nm)
S
S+P
S+P+PI
0
5
10
15
20
25
30
35
40
TIME OF INCUBATION AT 37° C (min)

FIG.8A

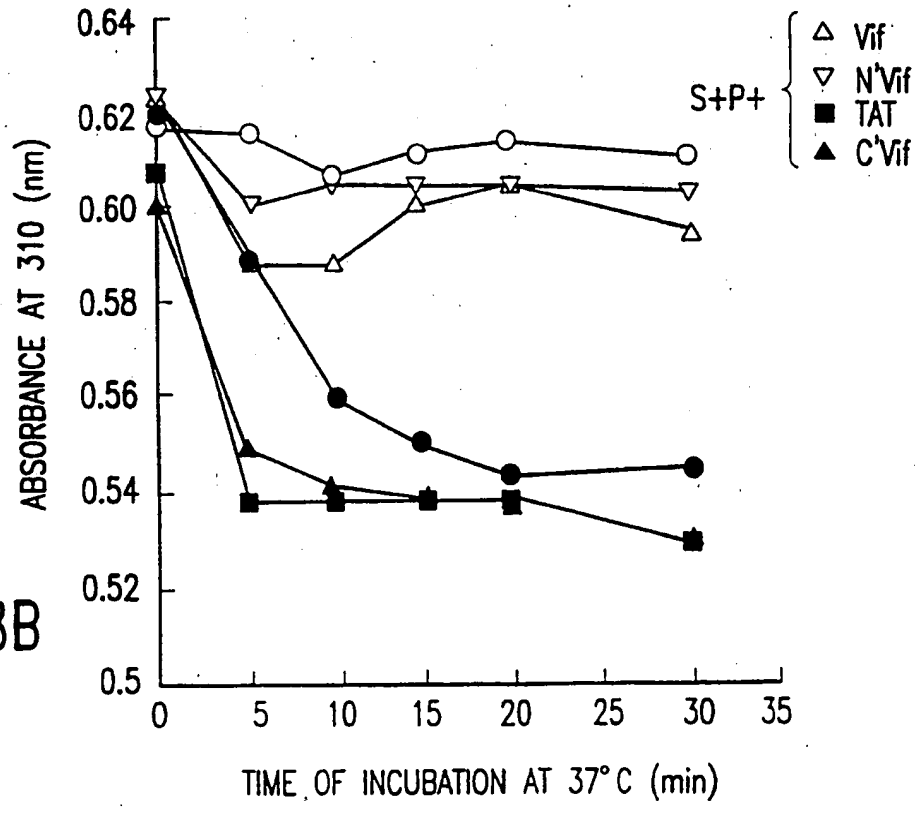

0.64
0.62
0.60
0.58
0.56
0.54
0.52
0.5
ABSORBANCE AT 310 (nm)
S+P+
Vif
N'Vif
TAT
C'Vif
0
5
10
15
20
25
30
35
TIME OF INCUBATION AT 37° C (min)

FIG.8B

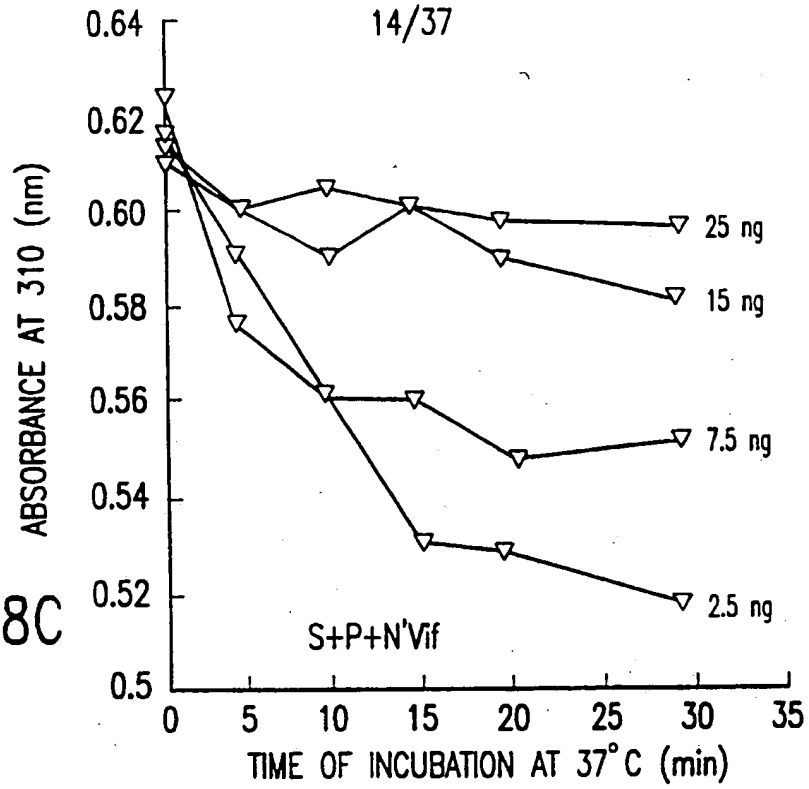

0.64
0.62
0.60
0.58
0.56
0.54
0.52
0.5
ABSORBANCE AT 310 (nm)
25 ng
15 ng
7.5 ng
2.5 ng
S+P+N'Vif
0
5
10
15
20
25
30
35
TIME OF INCUBATION AT 37°C (min)

FIG.8C

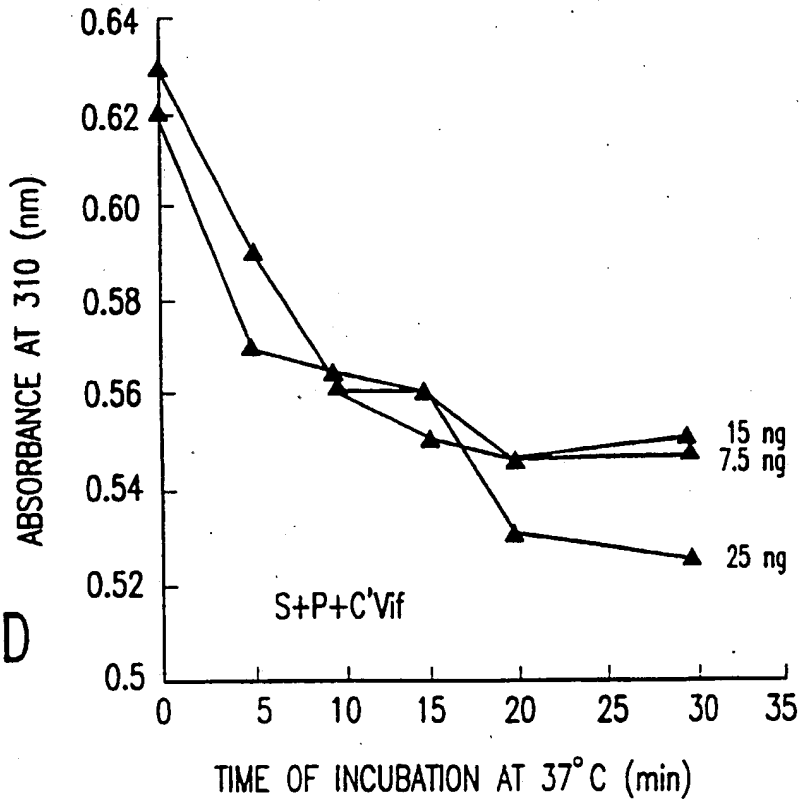

0.64
0.62
0.60
0.58
0.56
0.54
0.52
0.5
ABSORBANCE AT 310 (nm)
15 ng
7.5 ng
25 ng
S+P+C'Vif
0
5
10
15
20
25
30
35
TIME OF INCUBATION AT 37°C (min)

FIG.8D

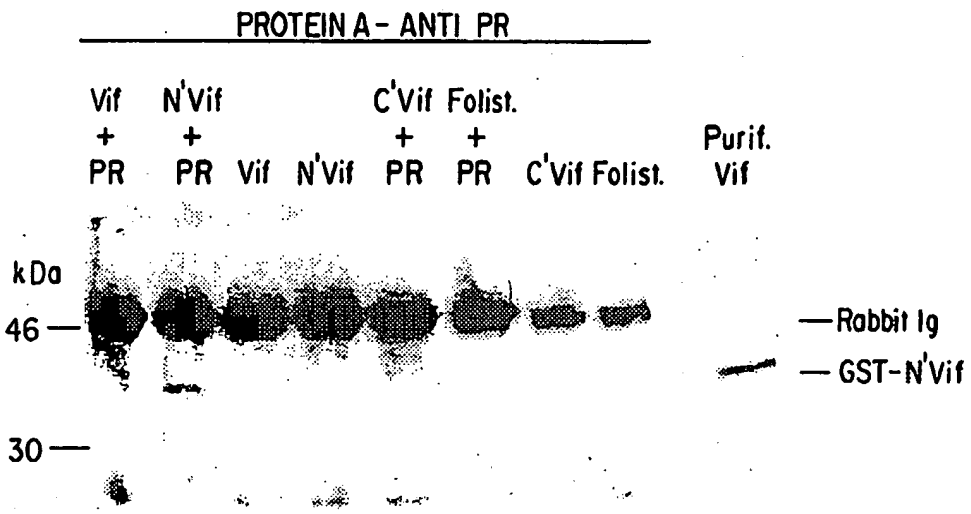

PROTEIN A - ANTI PR
Vif + PR
N'Vif + PR
Vif
N'Vif
C'Vif + PR
Folist. + PR
C'Vif
Folist.
Purif. Vif
kDa
46
30
Rabbit Ig
GST-N'Vif

FIG.9A

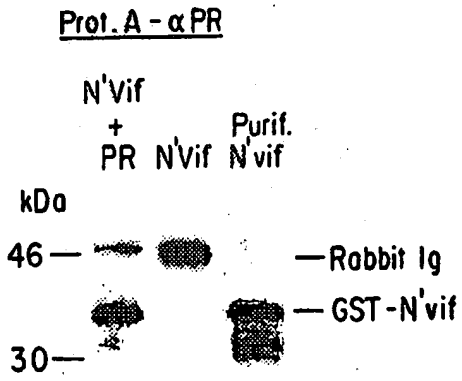

Prot. A - αPR
N'Vif + PR
N'Vif
Purif. N'vif
kDa
46
30
Rabbit Ig
GST-N'vif

FIG.9B

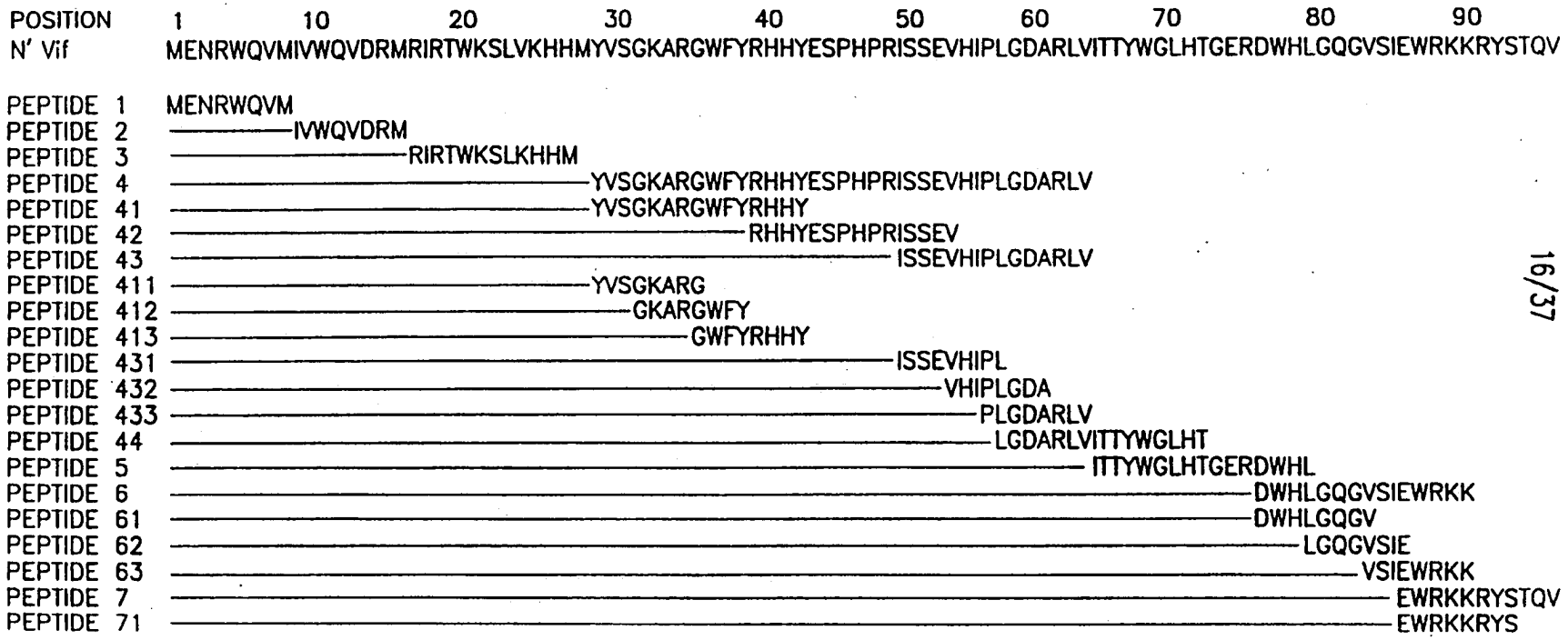

POSITION 1 10 20 30 40 50 60 70 80 90
N' Vif MENRWQVMIVWQVDRMRIRTWKSLVKHHMYVSGKARGWFYRHHYESPHPRISSEVHIPLGDARLVITTYWGLHTGERDWHLGQGVSIEWRKKRYSTQV
PEPTIDE 1 MENRWQVM
PEPTIDE 2 IVWQVDRM
PEPTIDE 3 RIRTWKSLKHHM
PEPTIDE 4 YVSGKARGWFYRHHYESPHPRISSEVHIPLGDARLV
PEPTIDE 41 YVSGKARGWFYRHHY
PEPTIDE 42 RHHYESPHPRISSEV
PEPTIDE 43 ISSEVHIPLGDARLV
PEPTIDE 411 YVSGKARG
PEPTIDE 412 GKARGWFY
PEPTIDE 413 GWFYRHHY
PEPTIDE 431 ISSEVHIPL
PEPTIDE 432 VHIPLGDA
PEPTIDE 433 PLGDARLV
PEPTIDE 44 LGDARLVITTYWGLHT
PEPTIDE 5 ITTYWGLHTGERDWHL
PEPTIDE 6 DWHLGQGVSIEWRKK
PEPTIDE 61 DWHLGQGV
PEPTIDE 62 LGQGVSIE
PEPTIDE 63 VSIEWRKK
PEPTIDE 7 EWRKKRYSTQV
PEPTIDE 71 EWRKKRYS

FIG.10A

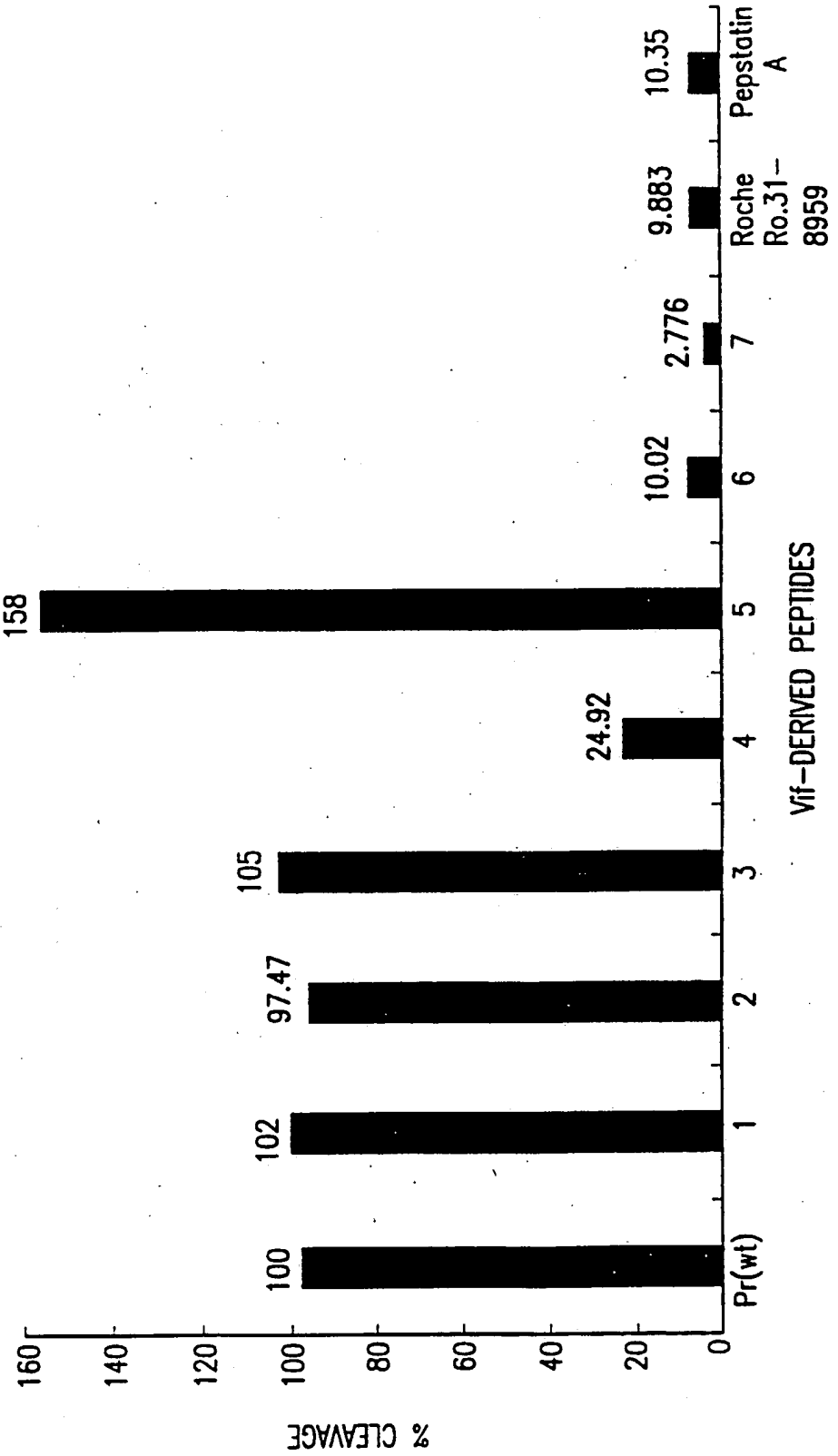

% CLEAVAGE
160
140
120
100
80
60
40
20
0
100
102
97.47
105
24.92
158
10.02
2.776
9.883
10.35
Pr(wt)
1
2
3
4
5
6
7
Roche Ro.31-8959
Pepstatin A
Vif-DERIVED PEPTIDES

FIG.10B

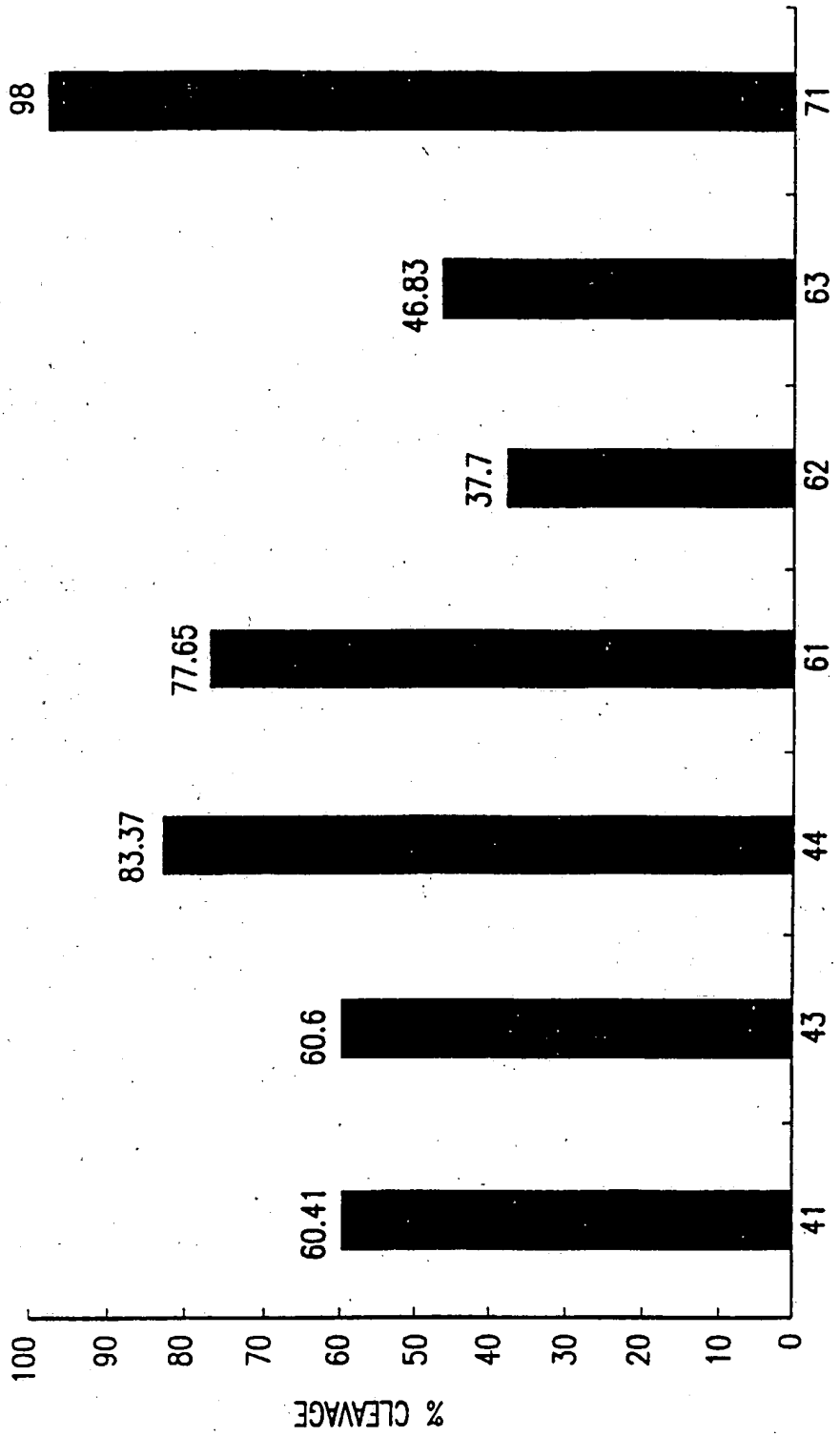

% CLEAVAGE
100
90
80
70
60
50
40
30
20
10
0
98
46.83
37.7
77.65
83.37
60.6
60.41
71
63
62
61
44
43
41
Vif-DERIVED PEPTIDES

FIG.10C

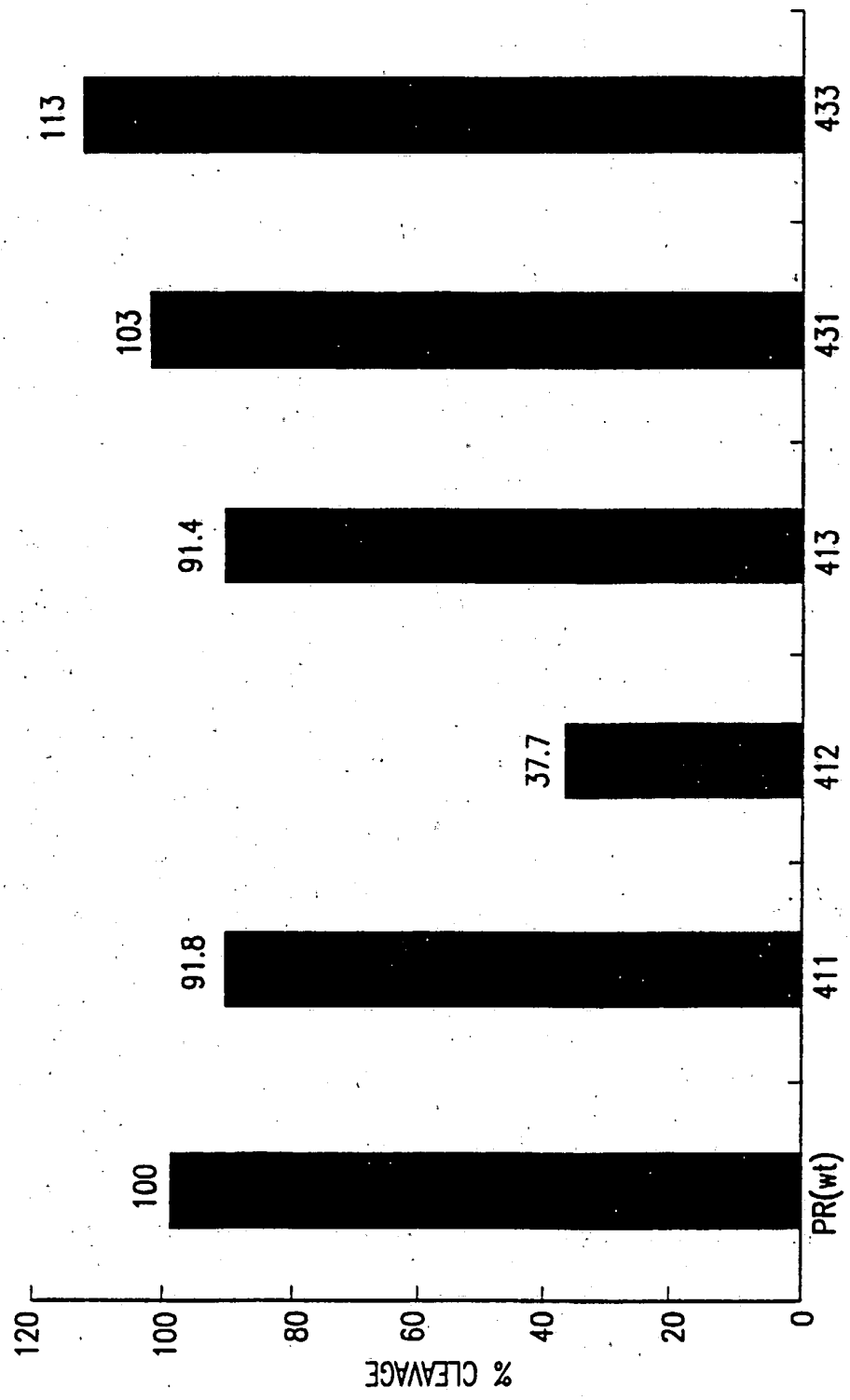

% CLEAVAGE
120
100
80
60
40
20
0
100
91.8
37.7
91.4
103
113
PR(wt)
411
412
413
431
433
Vif-DERIVED PEPTIDES

FIG.10D

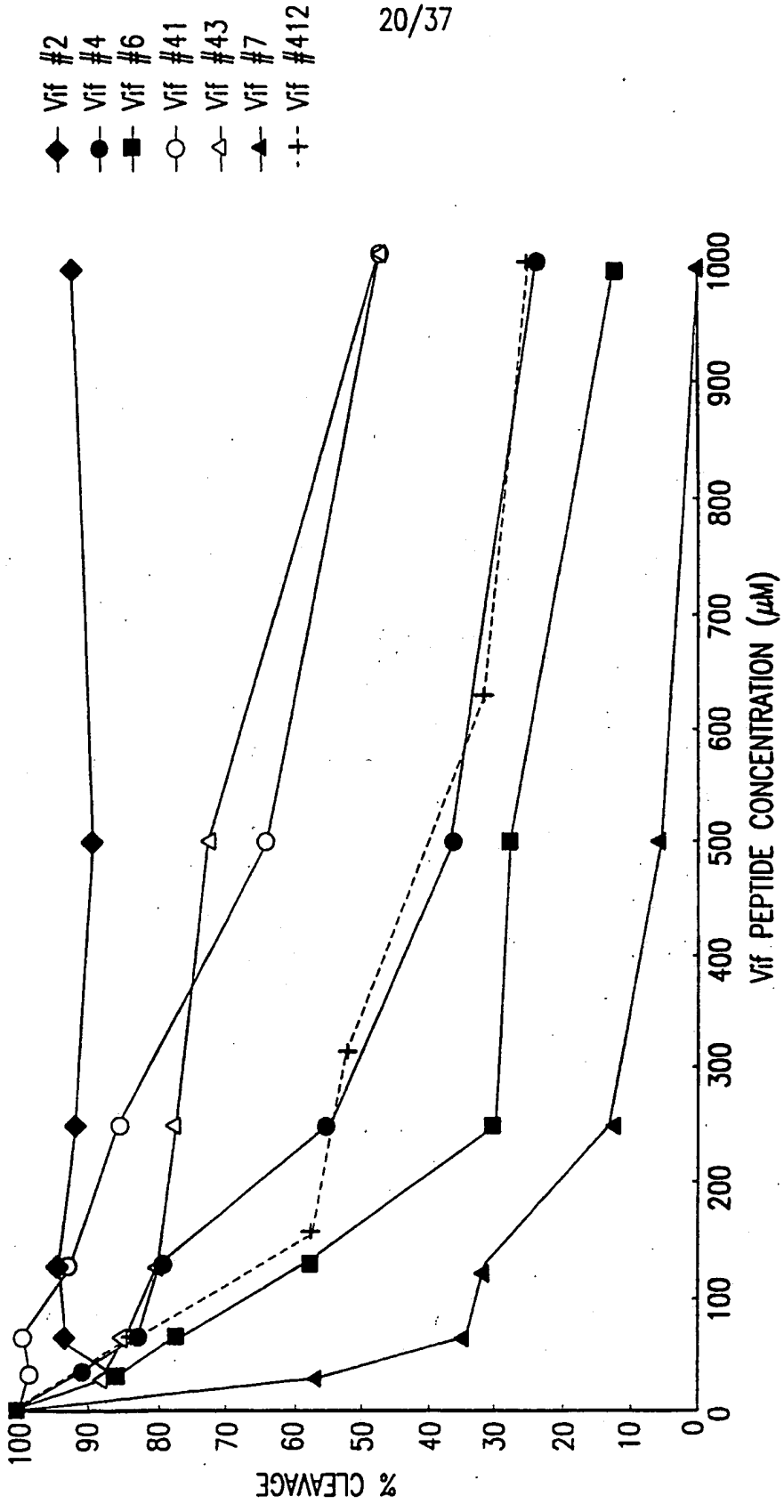

Vif #2
Vif #4
Vif #6
Vif #41
Vif #43
Vif #7
Vif #412
% CLEAVAGE
100
90
80
70
60
50
40
30
20
10
0
0
100
200
300
400
500
600
700
800
900
1000
Vif PEPTIDE CONCENTRATION (μM)

FIG.10E

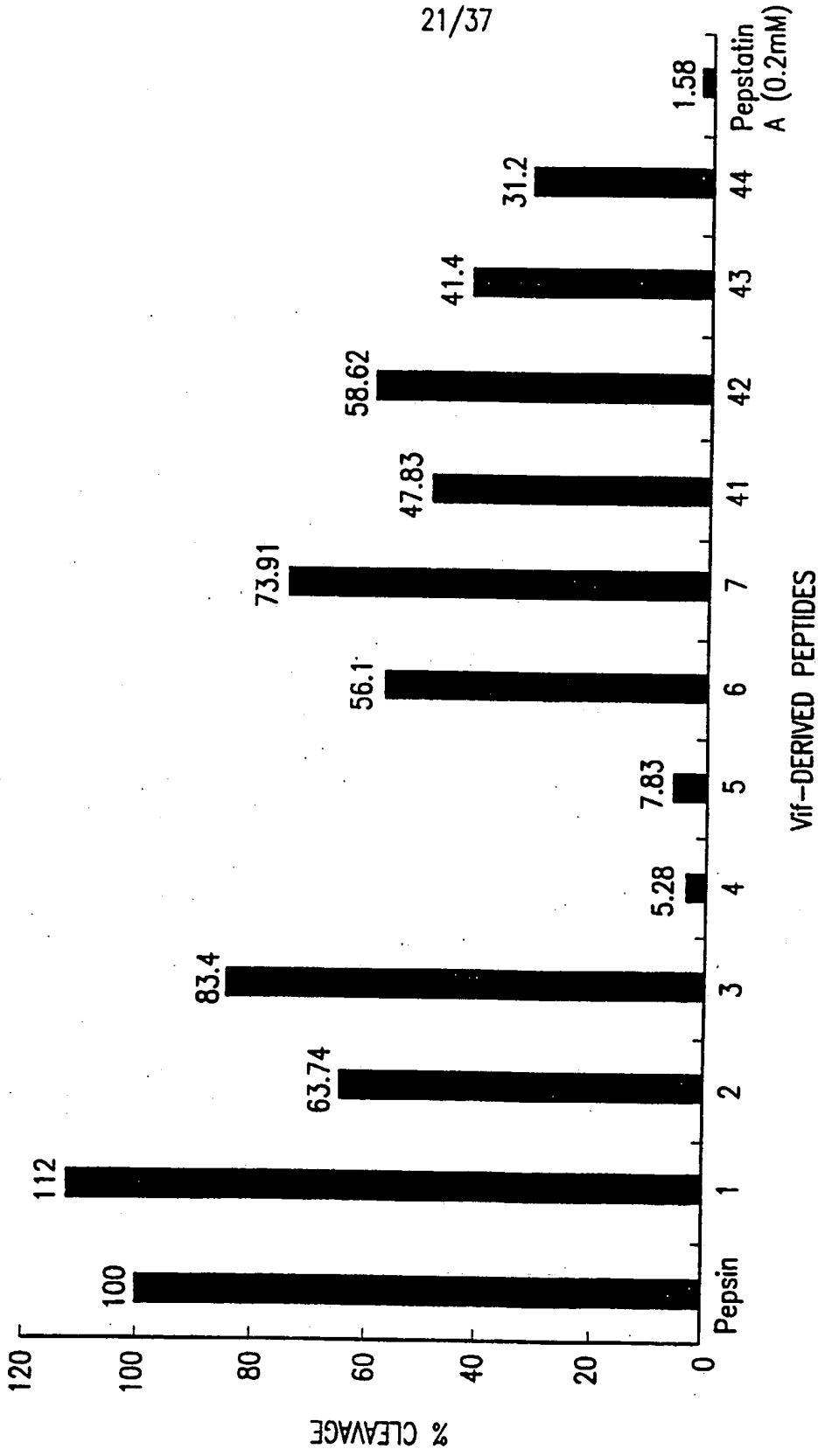

% CLEAVAGE
120
100
80
60
40
20
0
Pepsin
100
112
63.74
83.4
5.28
7.83
56.1
73.91
47.83
58.62
41.4
31.2
1.58
Pepstatin A (0.2mM)
1
2
3
4
5
6
7
41
42
43
44
Vif-DERIVED PEPTIDES

FIG.10F

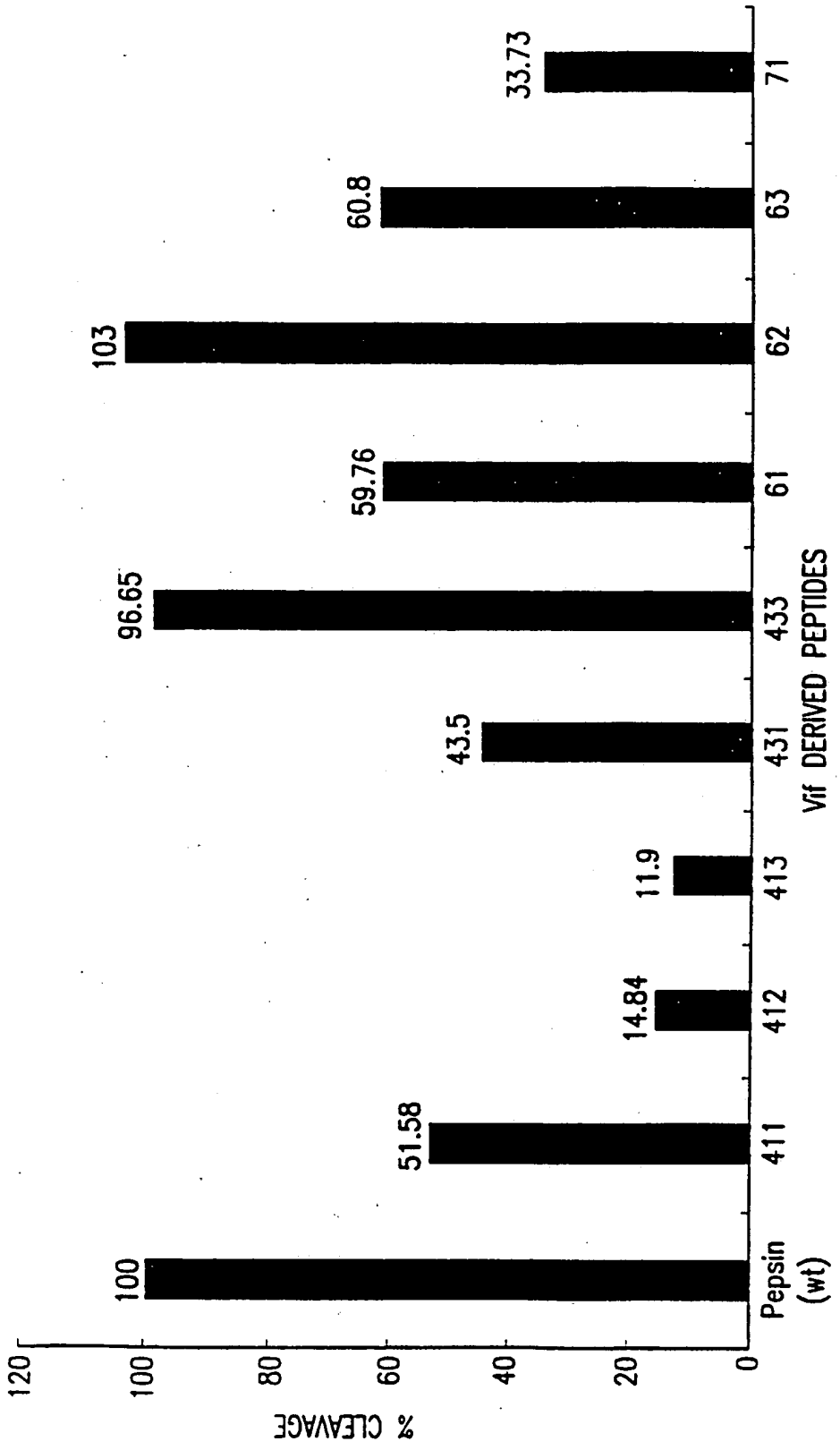

% CLEAVAGE
120
100
80
60
40
20
0
100
51.58
14.84
11.9
43.5
96.65
59.76
103
60.8
33.73
Pepsin (wt)
411
412
413
431
433
61
62
63
71
Vif DERIVED PEPTIDES

FIG.10G

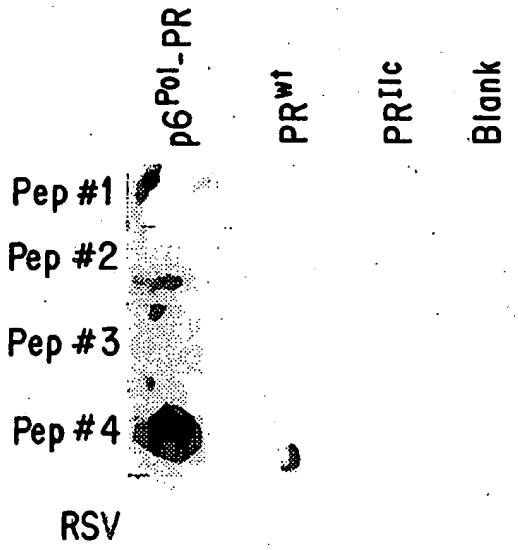


FIG.11A

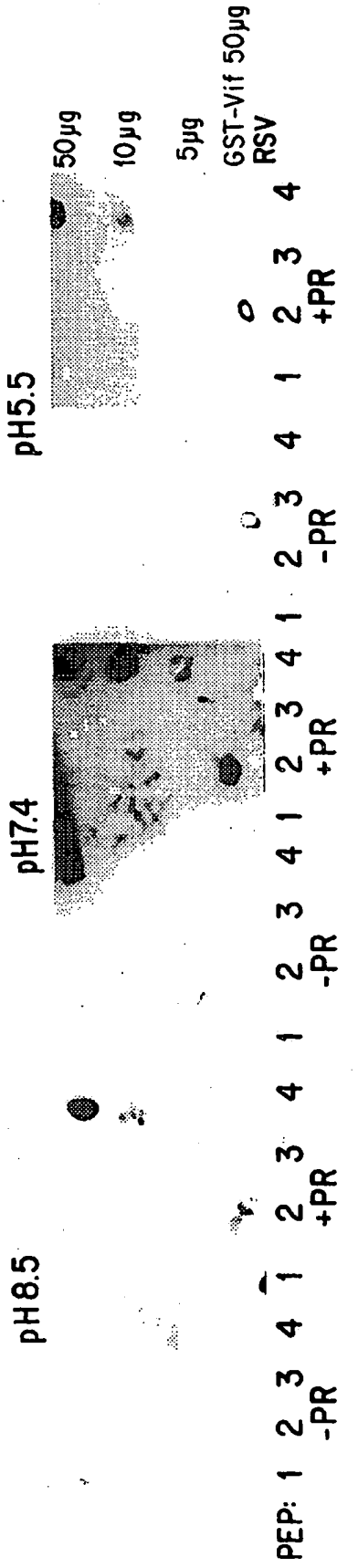

50µg
10µg
5µg
GST-Vif 50µg
RSV
pH5.5
pH7.4
pH8.5
PEP: 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
-PR +PR -PR +PR
0

FIG.11B

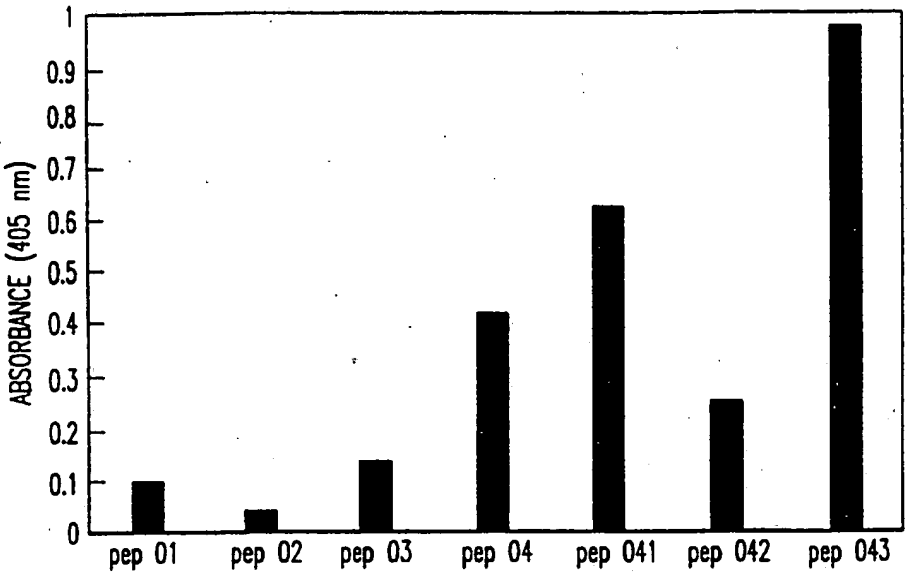


FIG.12A

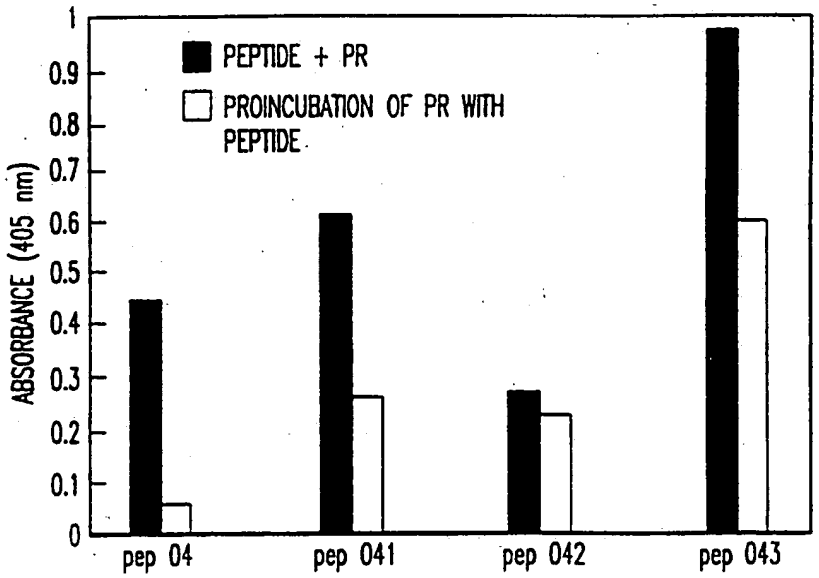


FIG.12B

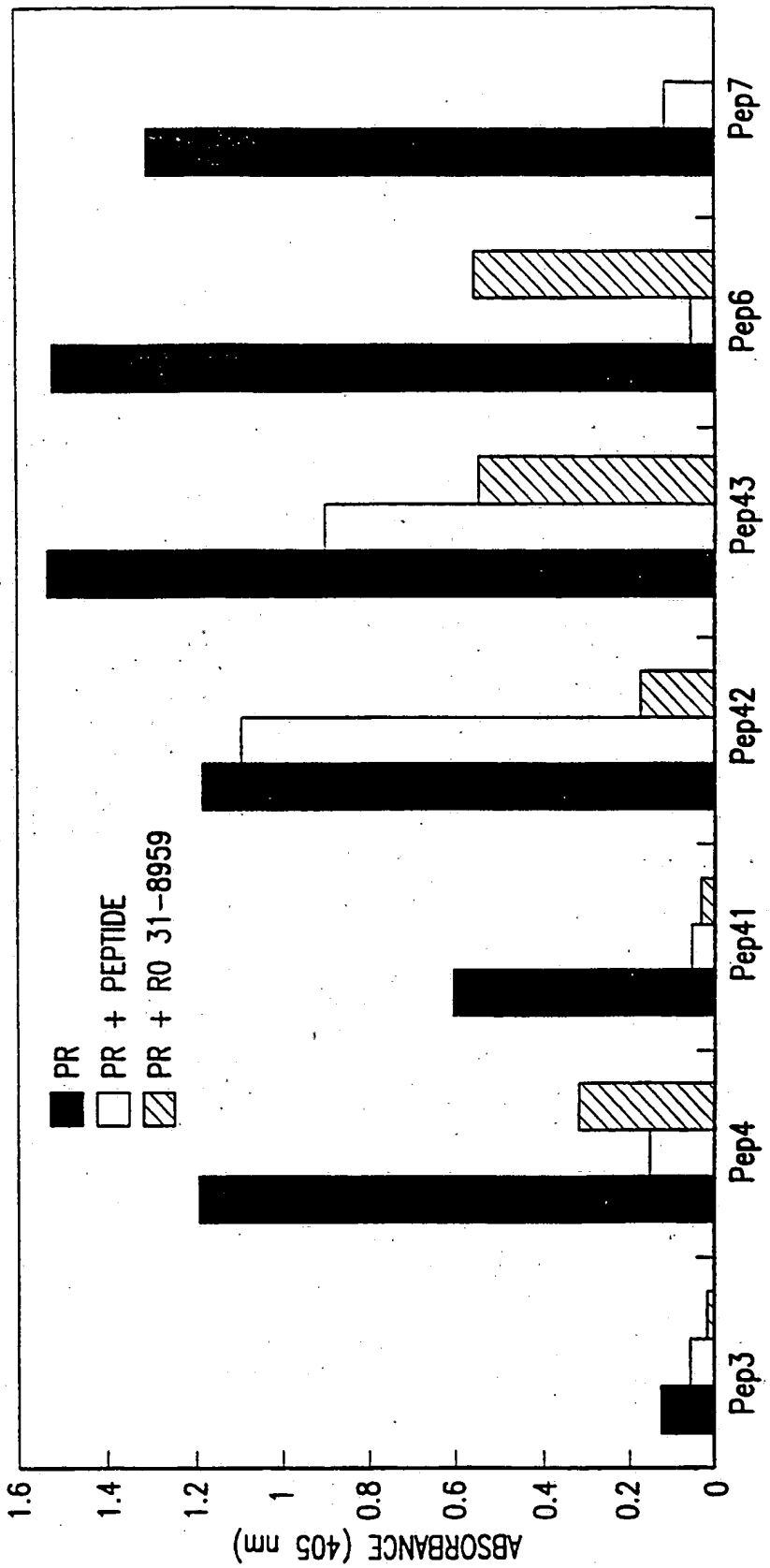

PR
PR + PEPTIDE
PR + RO 31-8959
Pep3
Pep4
Pep41
Pep42
Pep43
Pep6
Pep7
ABSORBANCE (405 nm)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6

FIG.12C

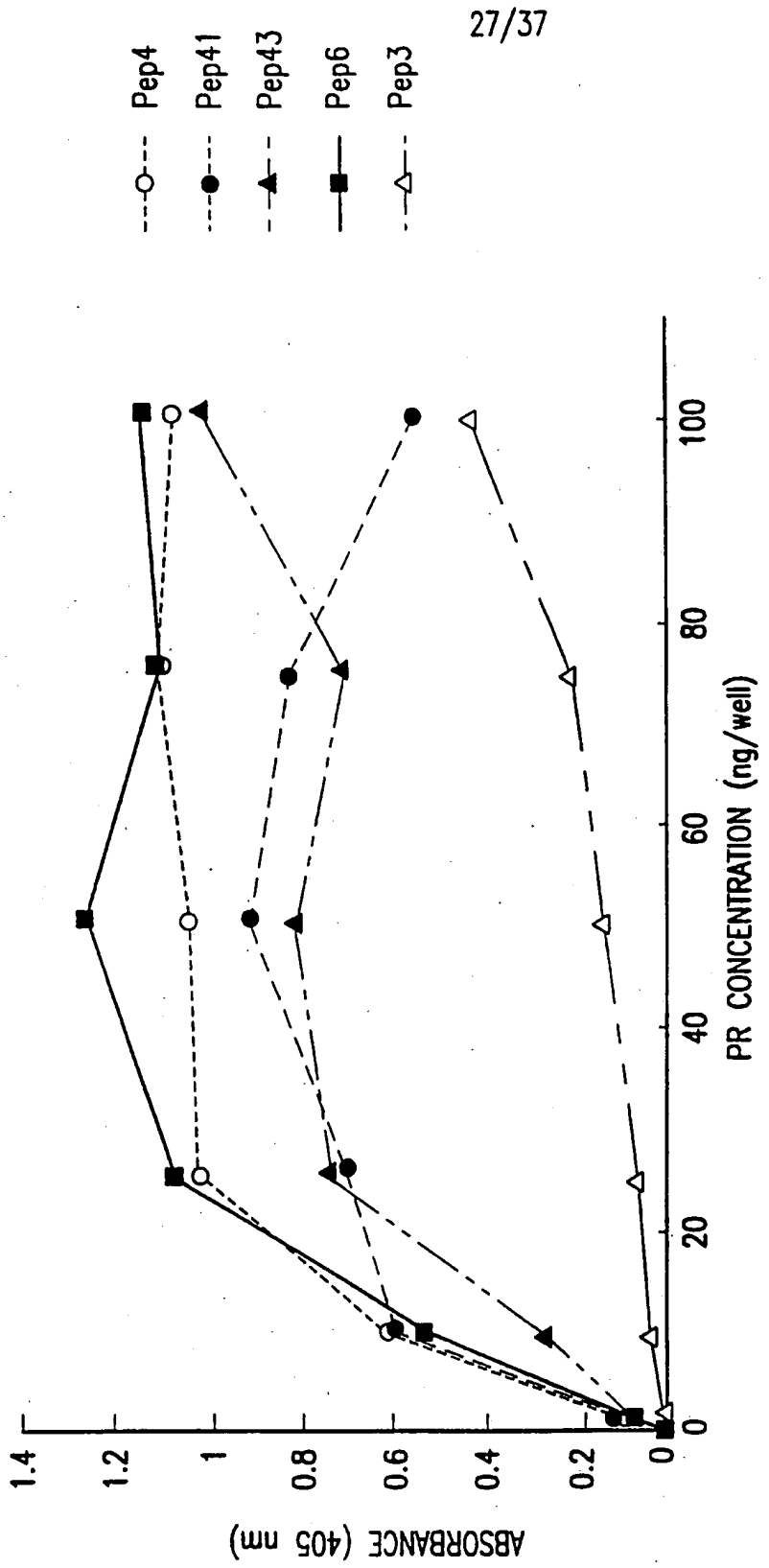

Pep4
Pep41
Pep43
Pep6
Pep3
ABSORBANCE (405 nm)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
0
20
40
60
80
100
PR CONCENTRATION (ng/well)

FIG.12D

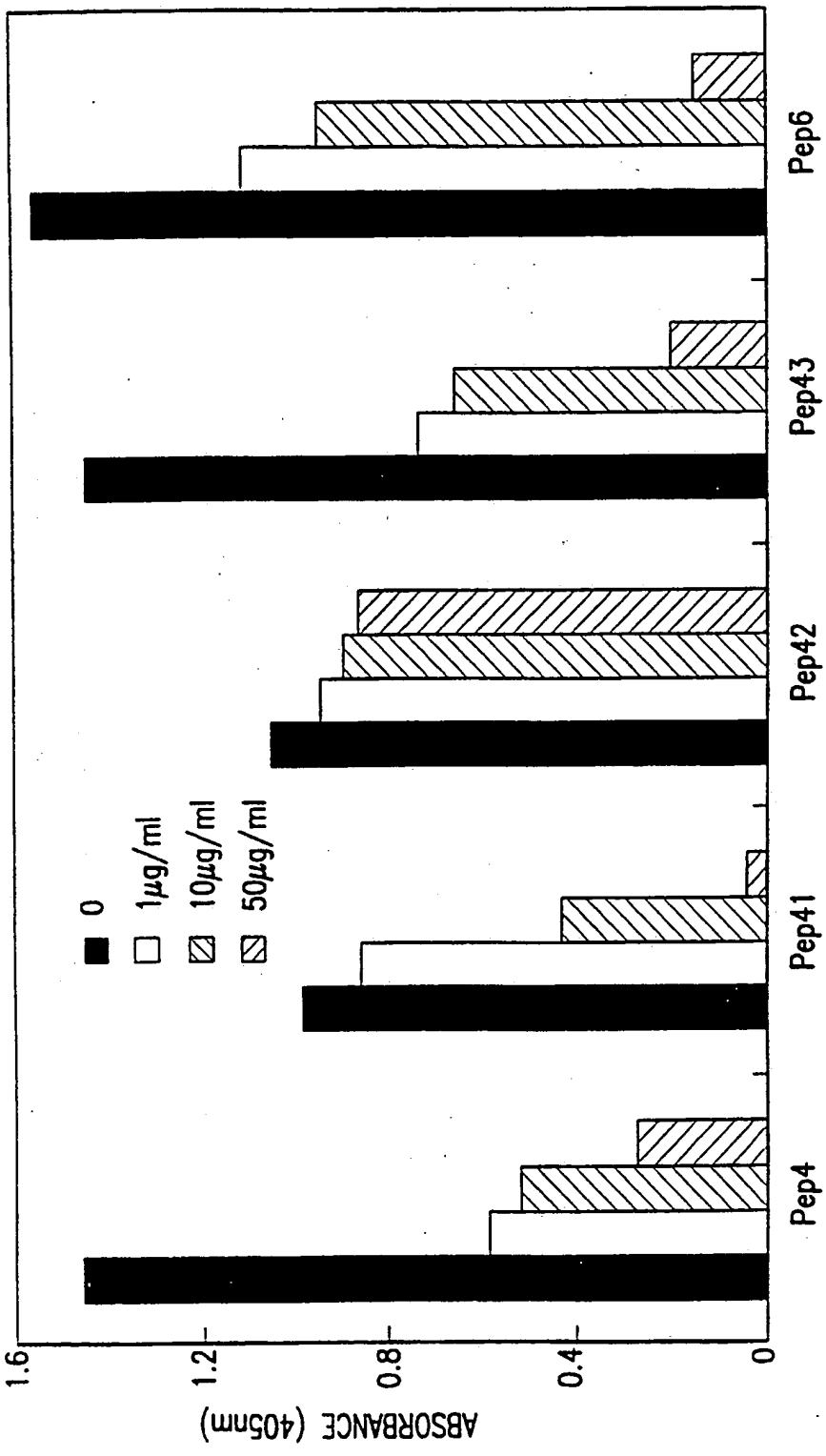

ABSORBANCE (405nm)
1.6
1.2
0.8
0.4
0
Pep4
Pep41
Pep42
Pep43
Pep6
0
1μg/ml
10μg/ml
50μg/ml

FIG.12E

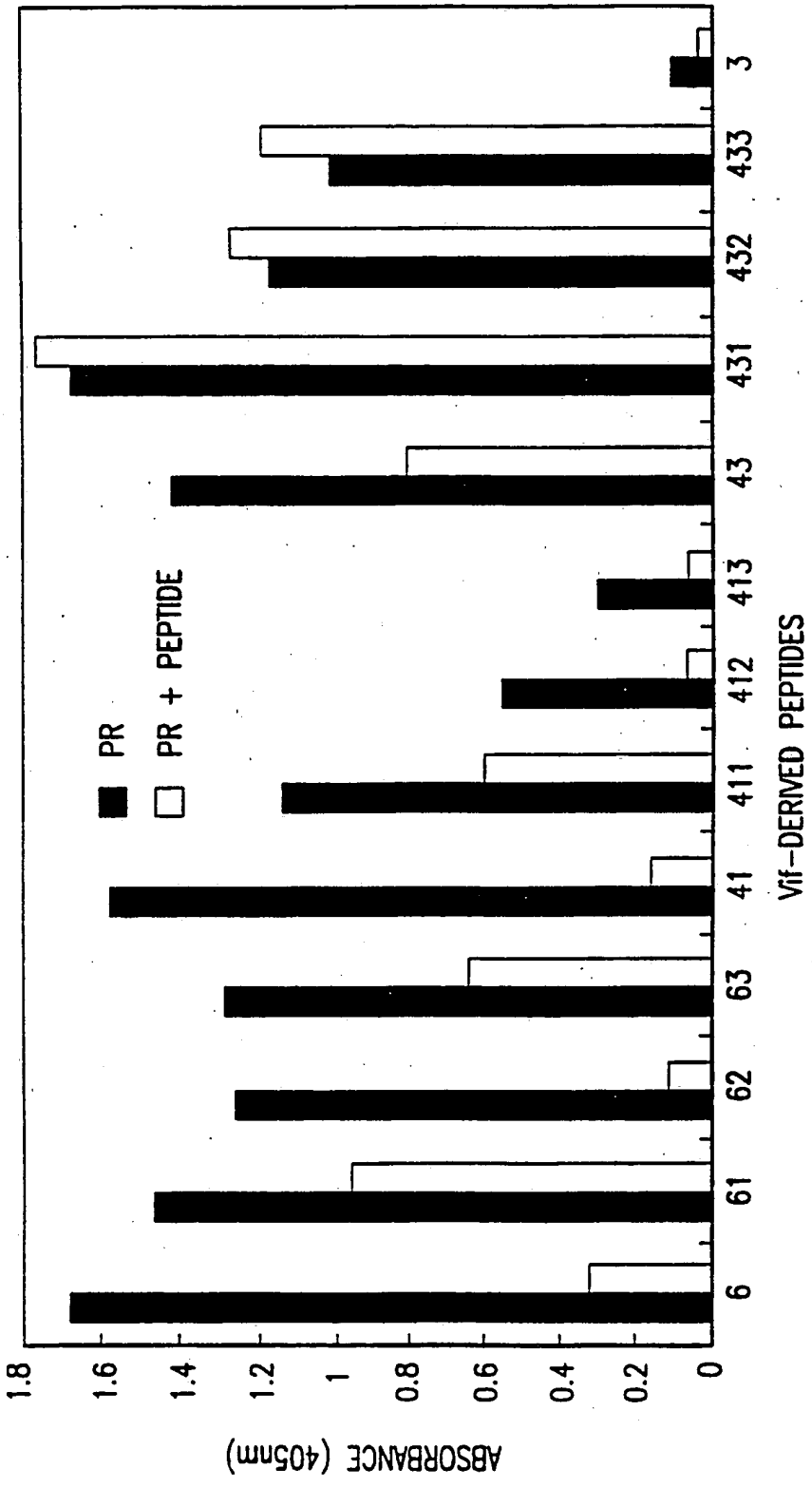

ABSORBANCE (405nm)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
PR
PR + PEPTIDE
6
61
62
63
41
411
412
413
43
431
432
433
3
Vif-DERIVED PEPTIDES

FIG.12F

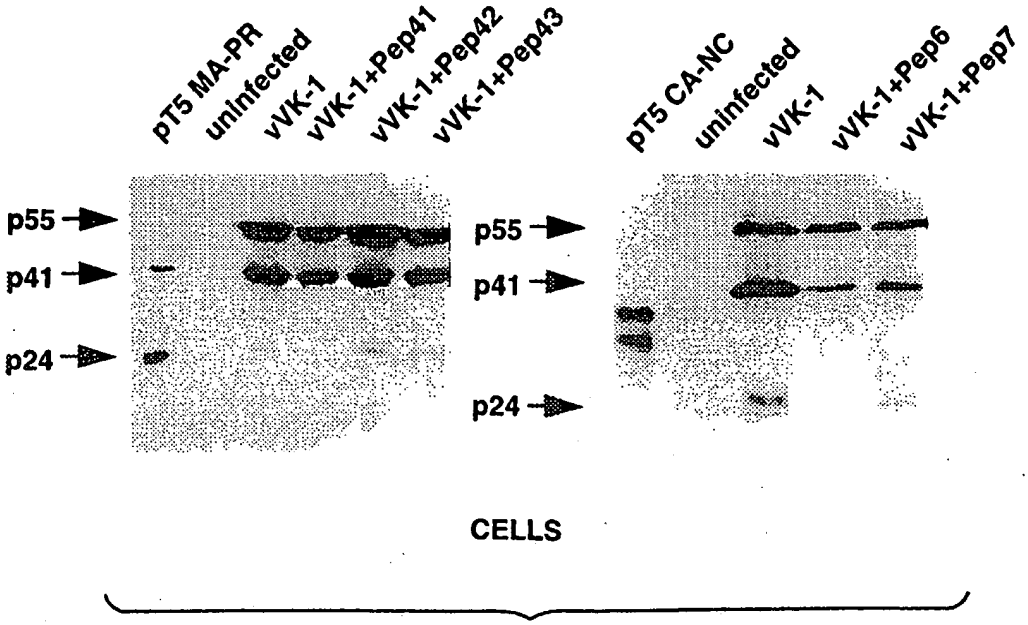


FIG.13A

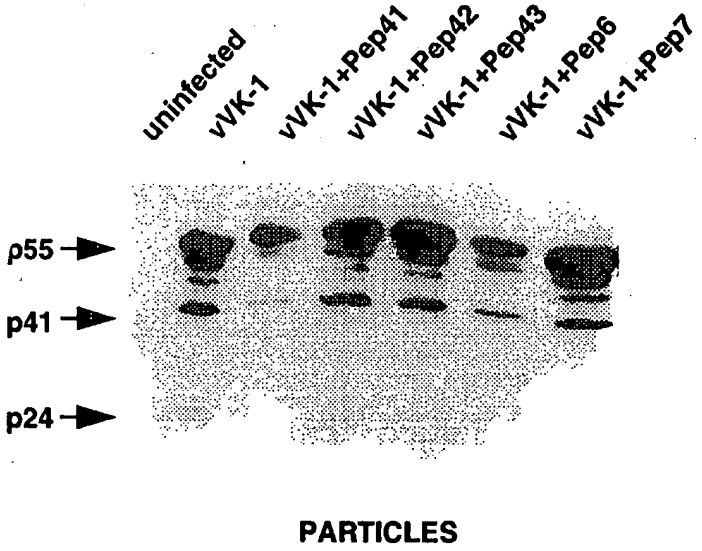


FIG.13B

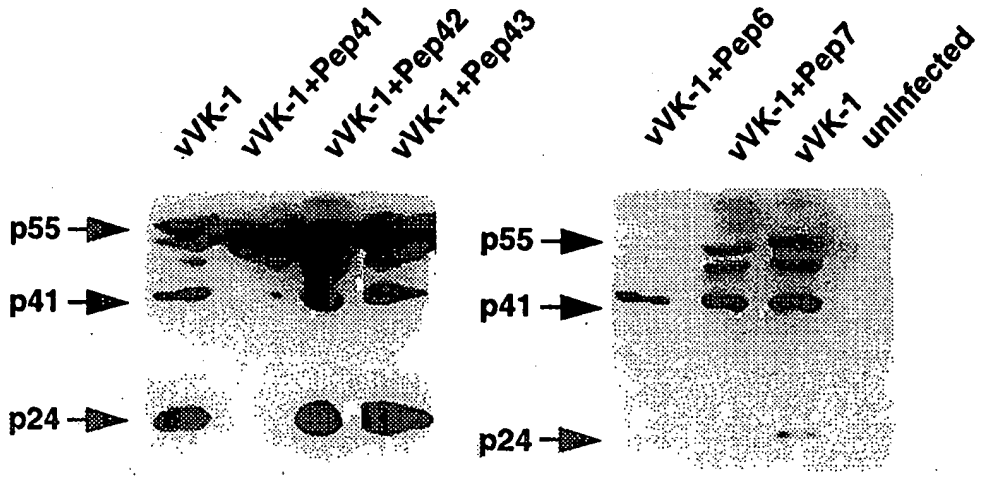


FIG. 13C

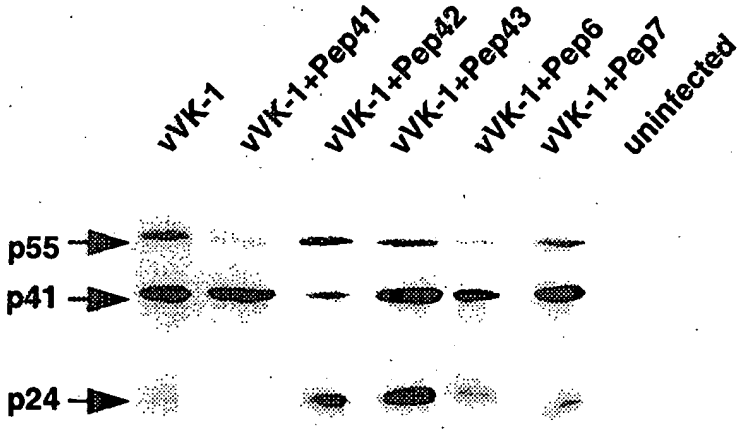


FIG. 13D

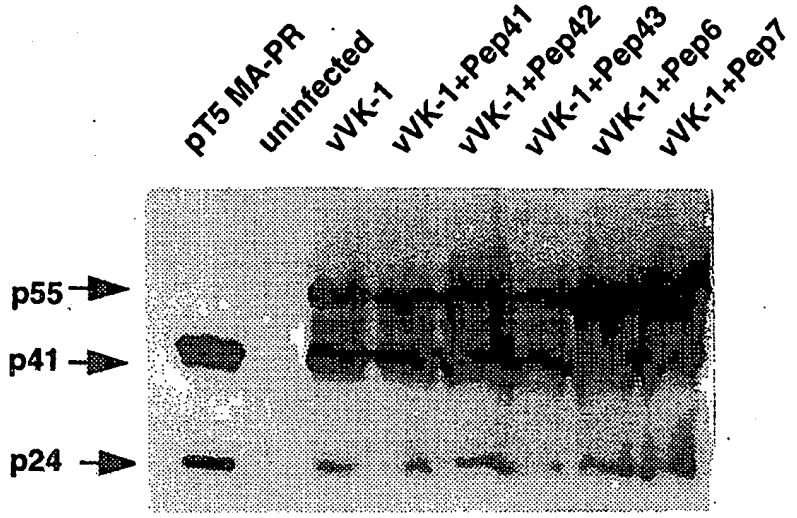


FIG.13E

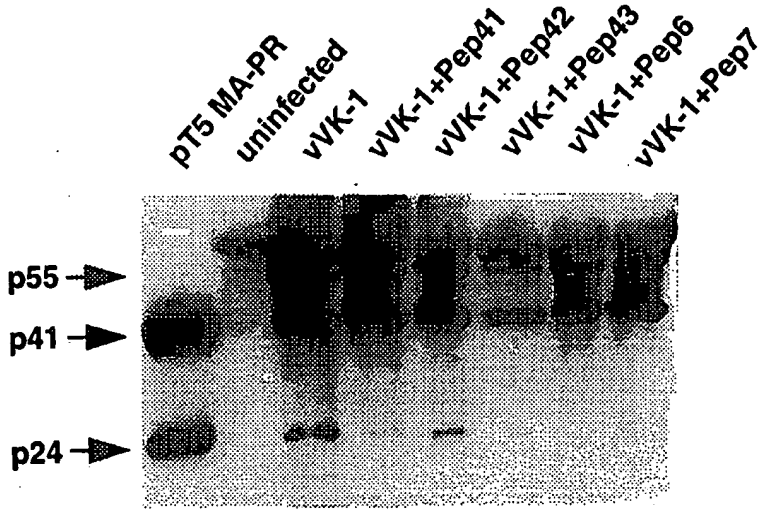


FIG.13F

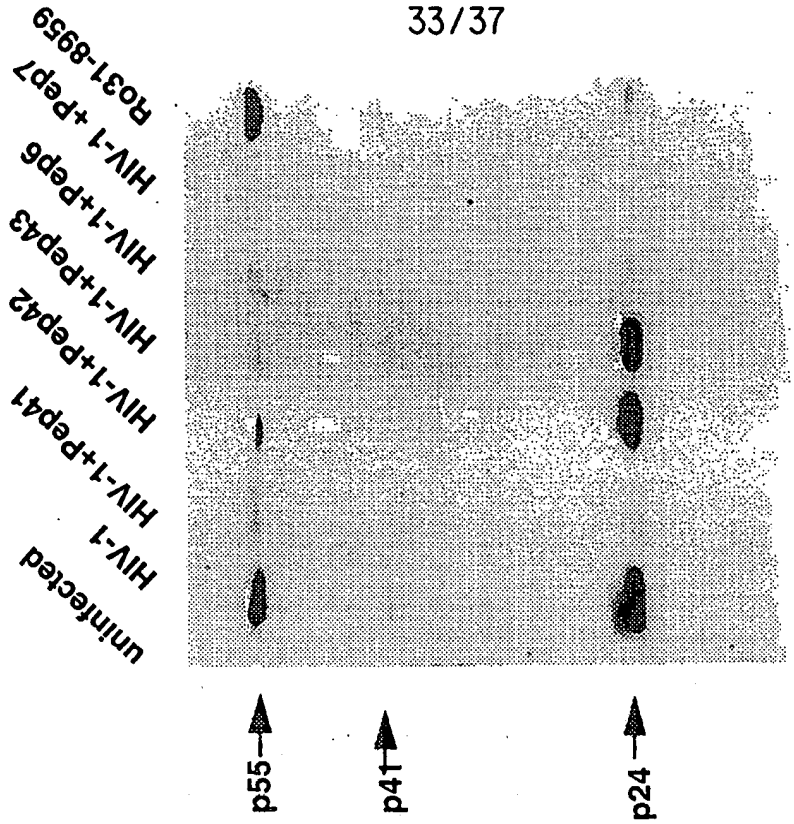


FIG.14B

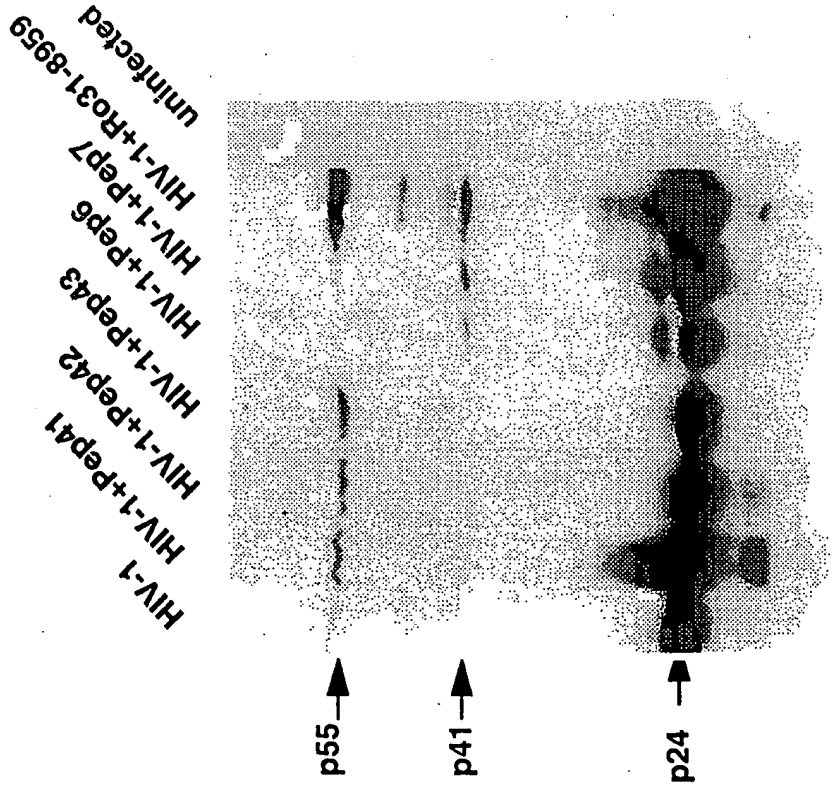


FIG.14A

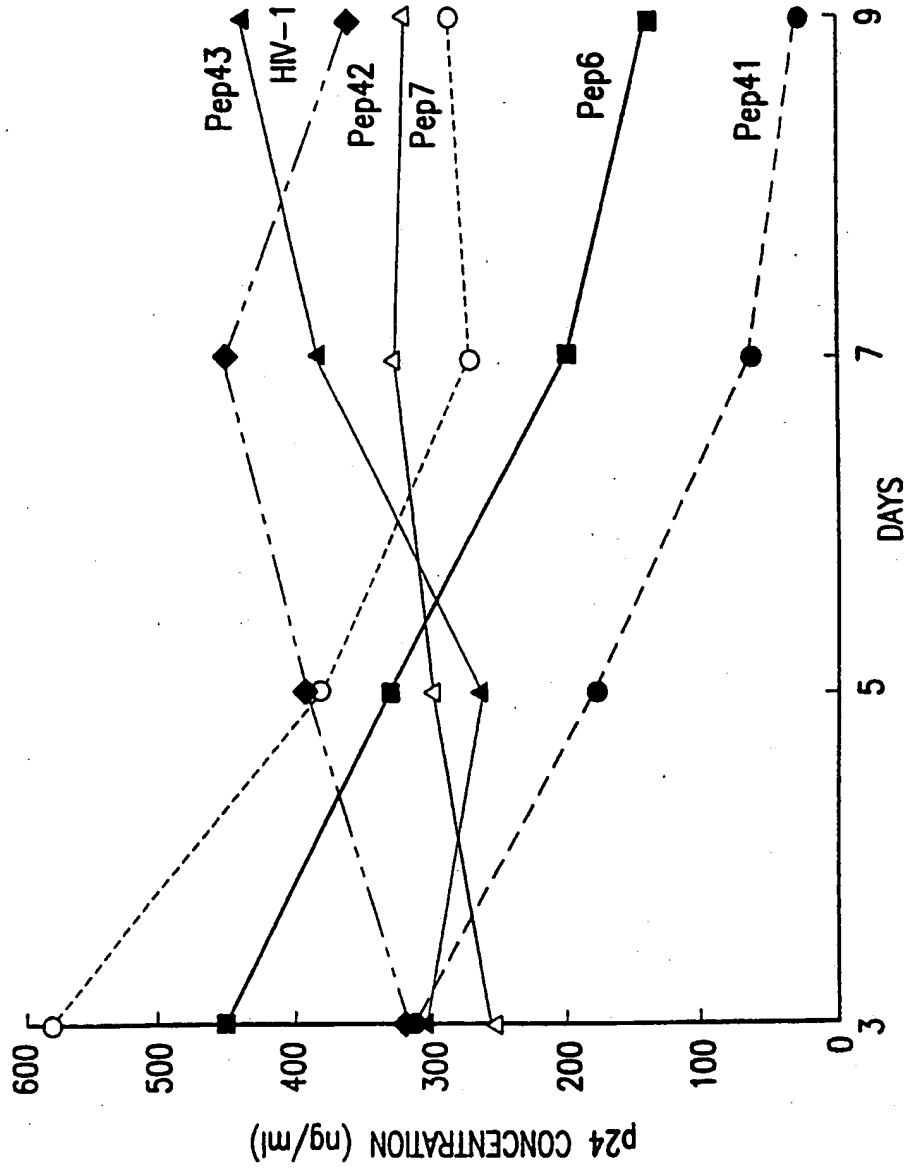

Pep43
HIV-1
Pep42
Pep7
Pep6
Pep41
600
500
400
300
200
100
0
3
5
7
9
DAYS
p24 CONCENTRATION (ng/ml)

FIG.15A

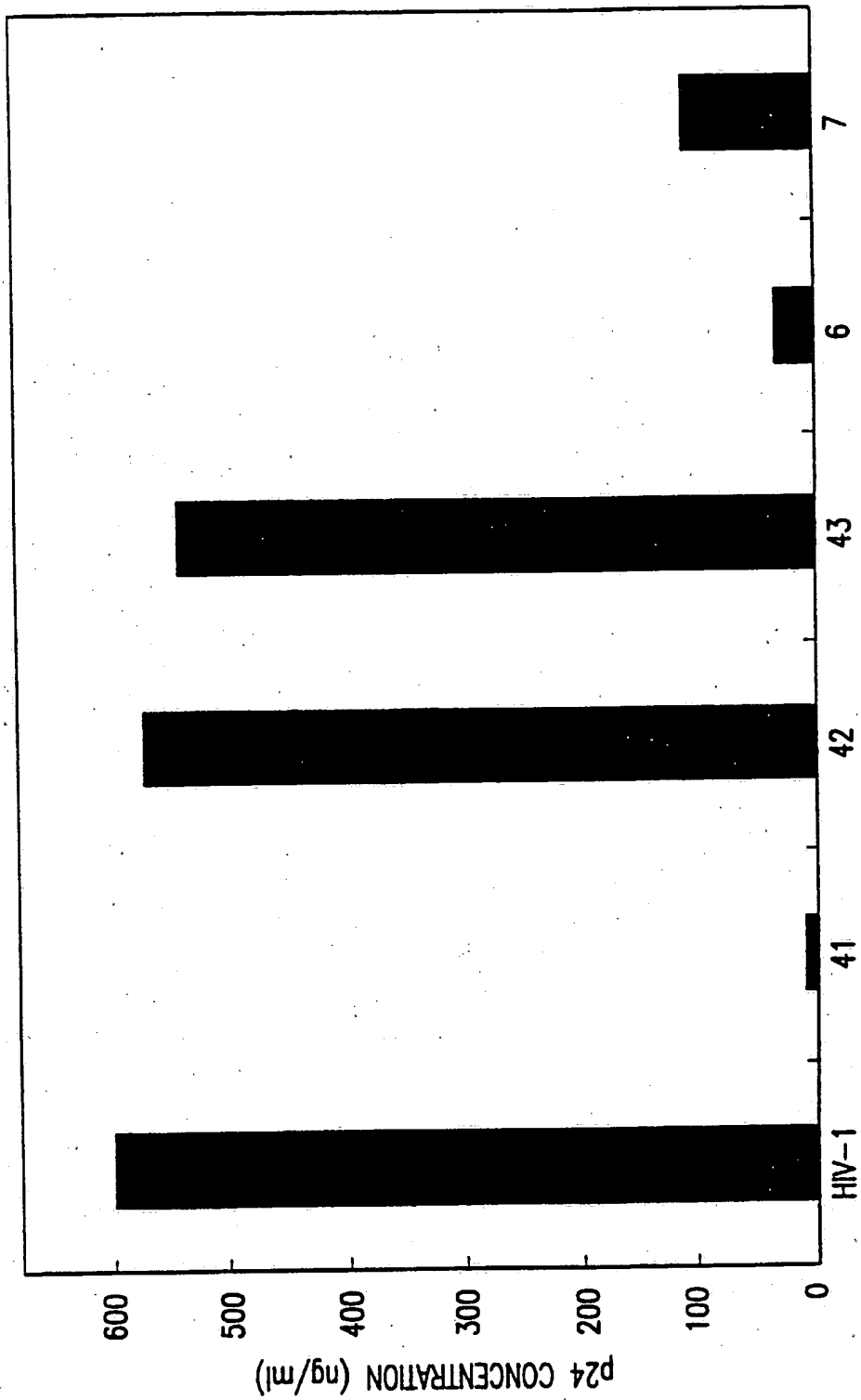

p24 CONCENTRATION (ng/ml)
0
100
200
300
400
500
600
HIV-1
41
42
43
6
7
Vif-DERIVED PEPTIDES

FIG.15B

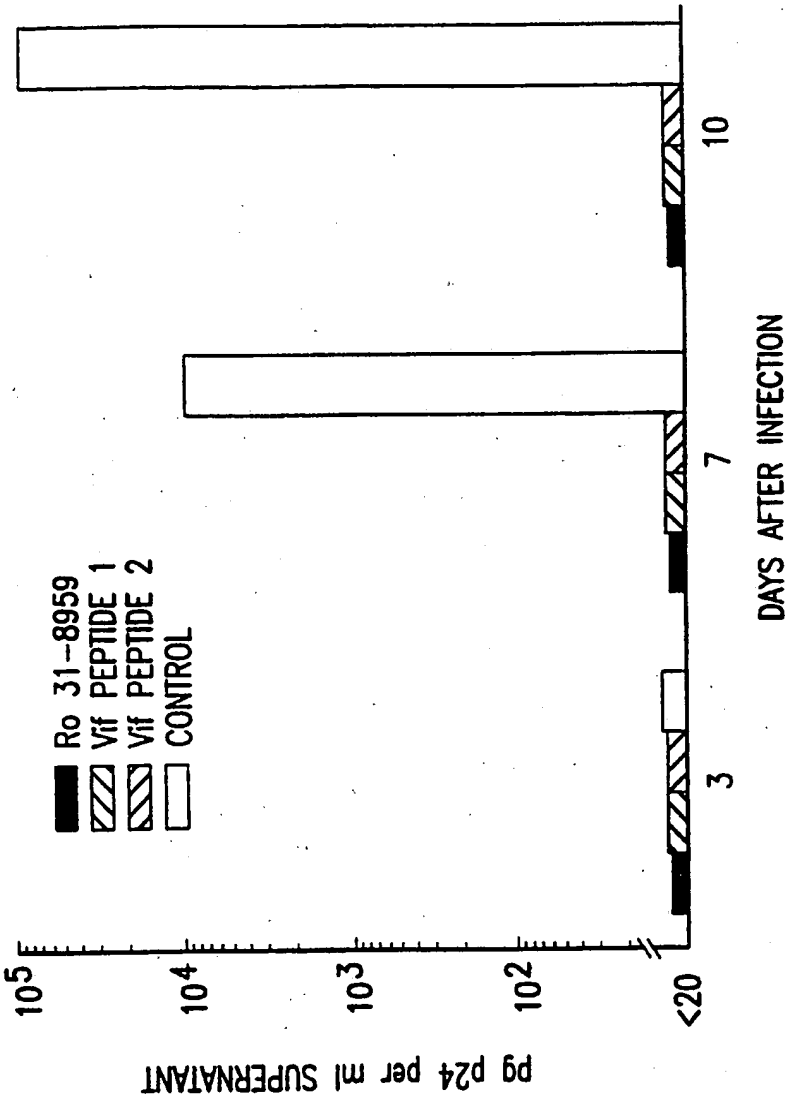

Ro 31-8959
Vif PEPTIDE 1
Vif PEPTIDE 2
CONTROL
10
7
3
DAYS AFTER INFECTION
10^5
10^4
10^3
10^2
<20
pg p24 per ml SUPERNATANT

FIG.16A

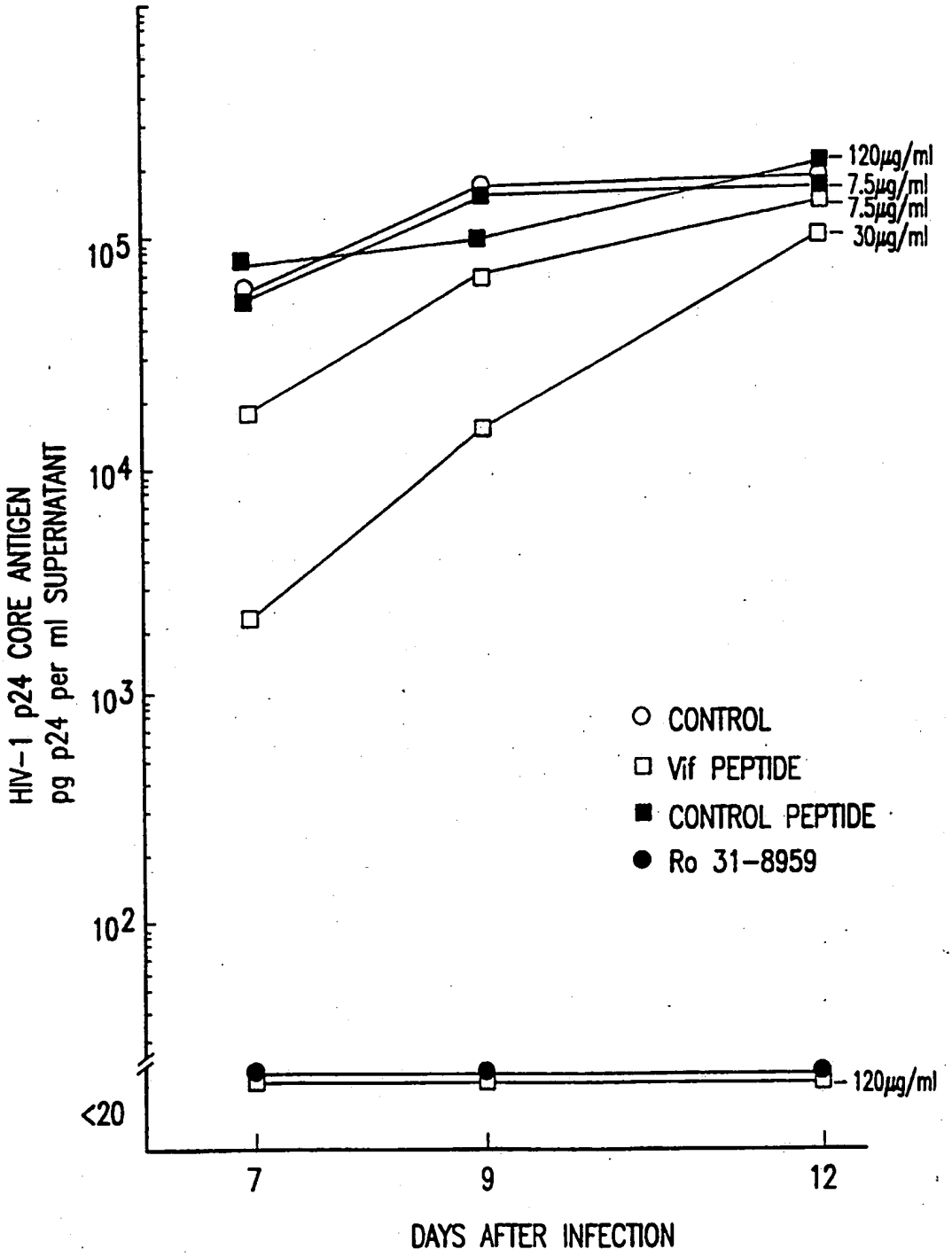

HIV-1 p24 CORE ANTIGEN
pg p24 per ml SUPERNATANT
10^5
10^4
10^3
10^2
<20
120µg/ml
7.5µg/ml
7.5µg/ml
30µg/ml
120µg/ml
CONTROL
Vif PEPTIDE
CONTROL PEPTIDE
Ro 31-8959
7
9
12
DAYS AFTER INFECTION

FIG.16B

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/10307

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/16 A61K38/55

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 330 359 A (BIO RAD LABORATORIES) 30 August 1989 see peptides 420 and 421 on page 7 see page 3, line 20 - line 28 see page 7, line 49 - line 55 see page 8, line 3 - line 10; claims ---	1-8
X	DE 41 41 970 A (CHARITE MED FAKULTAET) 17 June 1993 see column 2, line 50 - line 53; example 3 ---	1-8
X	EP 0 225 066 A (SPOFA VEREINIGTE PHARMA WERKE) 10 June 1987 see column 5, line 7 - line 11; example II --- -/--	1-8

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 October 1998	02/11/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Fuhr, C

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int ational Application No
PCT/US 98/10307

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	PATENT ABSTRACTS OF JAPAN vol. 096, no. 010, 31 October 1996 -& JP 08 151396 A (TEIJIN LTD), 11 June 1996 see pages 50 and 51 in corresponding patent publication JP8151396 see abstract	3
P,X	M. KOTLER ET AL.: "Human Imunodeficiency Virus Type 1 (HIV-1) Protein Vif Inhibits the Activity of HIV-1 Protease in Bacteria and In Vitro" JOURNAL OF VIROLOGY., vol. 71, no. 8, August 1997, pages 5774-5781, XP002080304 ICAN SOCIETY FOR MICROBIOLOGY US see the whole document	1-8

2

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/ 10307

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: As far as claims 7 and 8 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int tional Application No
PCT/US 98/10307

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0330359 A	30-08-1989	AU 3076189 A	31-08-1989
		JP 1308299 A	12-12-1989
DE 4141970 A	17-06-1993	DE 9117147 U	05-09-1996
EP 0225066 A	10-06-1987	CS 8508286 A	17-09-1987
		JP 62120399 A	01-06-1987
		US 4833072 A	23-05-1989